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# THE MULTIPLE NATURE OF VITAMIN B<sub>6</sub>: CRITIQUE OF METHODS FOR THE DETERMINATION OF THE COMPLEX AND ITS COMPONENTS

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(Received for publication, May 19, 1945)

Snell and collaborators (1-3) reported that amination or partial oxidation of pyridoxine yields compounds much more active for certain lactic acid bacteria than the original vitamin. Syntheses (4, 5) and microbiological assays (6) indicated that the active derivatives were pyridoxamine and pyridoxal. Hochberg, Melnick, and Oser (7) showed that pyridoxine is not the only compound in natural materials possessing vitamin B<sub>6</sub> activity for the rat and for microorganisms. In certain products only a small portion of vitamin B<sub>6</sub> activity could be attributed to pyridoxine, whereas in others this compound was practically the only member of the vitamin B<sub>6</sub> complex present. Snell (8) subsequently demonstrated by differential microbiological assays that pyridoxamine and pyridoxal are responsible for a considerable portion of the total vitamin B<sub>6</sub> activity of some natural materials and that these compounds are equivalent to pyridoxine in activity for the rat (9).

Most of the chemical and microbiological methods for the determination of vitamin B<sub>6</sub> were published before it was demonstrated that pyridoxine content and vitamin B<sub>6</sub> activity are not synonymous. Pyridoxine has been employed as the reference standard in vitamin B<sub>6</sub> assays. In the light of these more recent findings it was considered worth while to investigate the more promising methods in order to ascertain their reliability for measuring pyridoxine itself as well as other compounds with similar biological activity. Snell (8, 9) published the results of similar investigations with various microbiological assay procedures. However, some of his conclusions are open to question, particularly those dealing with the reliability of the differential microbiological techniques employed for fractionating the vitamin B<sub>6</sub> group into pyridoxine, pyridoxamine, and pyridoxal. In the present investigation physical, chemical, microbiological, and biological assay procedures were employed in a critical study of their applicability to pure systems and to extracts of biological materials. From the data accumulated, it has become apparent that there are still other factors in the vitamin B<sub>6</sub> group in addition to pyridoxine, pyridoxamine, and pyridoxal.

## EXPERIMENTAL

Vitamin B<sub>6</sub> and pyridoxine have been found in nature to be partly bound as complexes, resisting detection by microbiological (10, 11) and chemical (12, 7) assays. The rat is able to utilize the bound as well as the free forms (11, 7). Whereas some authors (12, 11, 7) have advocated strong acid hydrolysis for the liberation of the bound vitamin, one group of investigators (10) recommended solutions of low acidity for this purpose. In the present report studies were made of the stability of pyridoxine, pyridoxamine, and pyridoxal, and of other factors in the naturally occurring vitamin B<sub>6</sub> complex, when subjected to a variety of hydrolytic procedures.<sup>1</sup> These involved autoclaving the materials at 15 pounds pressure in 0.055 N H<sub>2</sub>SO<sub>4</sub> (10), autoclaving in 2 N H<sub>2</sub>SO<sub>4</sub> (11), and suspending the preparations in 4 N HCl at 100° (7).

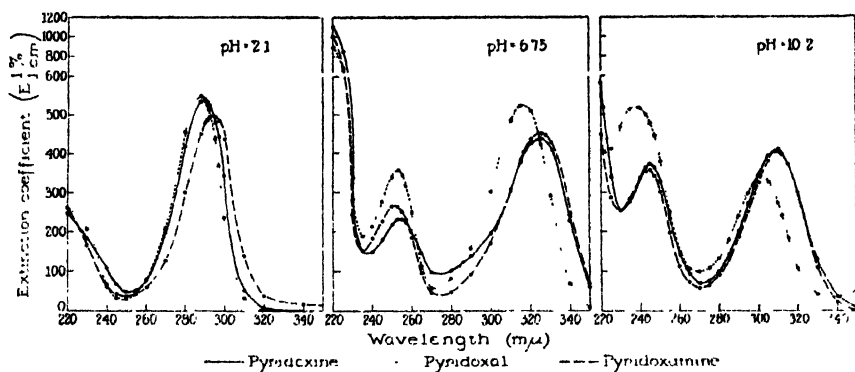


FIG. 1. Ultraviolet absorption curves of pyridoxine and derivatives in solutions at different pH values.

*Spectrophotometric Studies*—Investigators at the Merck laboratories (13, 14) have reported that pyridoxine shows a characteristic absorption spectrum which changes markedly with variations in pH. In Fig. 1 are presented the ultraviolet absorption curves<sup>2</sup> for pyridoxine, pyridoxal, and pyridoxamine solutions at three different hydrogen ion concentrations; the values plotted are those for the free bases. The molecular weights of these compounds are practically the same. The data for pyridoxine are in excellent agreement with those published by Stiller and associates (14).

<sup>1</sup> We are indebted to Dr. Karl Folkers, Merck and Company, Inc., Rahway, for supplying us with generous quantities of pyridoxamine dihydrochloride and pyridoxal hydrochloride.

<sup>2</sup> A Beckman spectrophotometer (15) with hydrogen discharge tube and quartz accessories was used for the measurements.

Each of the three compounds in acid solution (pH 2.1) exhibits a single absorption maximum, pyridoxamine at 292  $m\mu$ , pyridoxal and pyridoxine at 286 and 288  $m\mu$ , respectively. The "acid" band in each case is absent

TABLE I

*Ultraviolet Absorption Data on Pyridoxine and Related Compounds Subjected to Various Hydrolytic Procedures\**

Compound†	Hydrolytic procedure	Absorption maximum	$E_{1\text{cm}}^{1\%}$ maximum	Extinction ratios ( $E_{\text{max.}} = 1.00$ ) at								
				300 $m\mu$	310 $m\mu$	315 $m\mu$	320 $m\mu$	325 $m\mu$	330 $m\mu$	340 $m\mu$	350 $m\mu$	
Pyridoxine	None	325	438	0.42	0.70	0.86	0.97	1.00	0.94	0.53	0.15	
	0.055 N $\text{H}_2\text{SO}_4$ , 15 lbs. pressure, 90 min.	325	450	0.46	0.72	0.87	0.98	1.00	0.93	0.54	0.17	
	4 N $\text{HCl}$ , 100°, 60 min.	325	469	0.46	0.71	0.87	0.97	1.00	0.93	0.54	0.19	
	2 N $\text{H}_2\text{SO}_4$ , 15 lbs. pressure, 30 min.	325	487	0.50	0.75	0.88	0.97	1.00	0.93	0.56	0.17	
Pyridoxamine	None	325	454	0.40	0.69	0.85	0.97	1.00	0.96	0.55	0.16	
	0.055 N $\text{H}_2\text{SO}_4$ , 15 lbs. pressure, 90 min.	325	461	0.49	0.73	0.86	0.97	1.00	0.95	0.57	0.20	
	4 N $\text{HCl}$ , 100°, 60 min.	325	504	0.45	0.70	0.85	0.96	1.00	0.94	0.59	0.21	
	2 N $\text{H}_2\text{SO}_4$ , 15 lbs. pressure, 30 min.	325	512	0.47	0.70	0.84	0.95	1.00	0.95	0.60	0.23	
Pyridoxal	None	316	530	0.60	0.92	1.00	0.97	0.80	0.56	0.13	0.02	
	0.055 N $\text{H}_2\text{SO}_4$ , 15 lbs. pressure, 90 min.	316	542	0.62	0.92	1.00	0.97	0.82	0.63	0.16	0.05	
	4 N $\text{HCl}$ , 100°, 60 min.	316	633	0.67	0.93	0.99	0.97	0.85	0.62	0.24	0.13	
	2 N $\text{H}_2\text{SO}_4$ , 15 lbs. pressure, 30 min.	316	591	0.65	0.93	1.00	0.97	0.82	0.58	0.23	0.08	

\* The solutions were buffered at pH 6.75 for the spectrophotometric measurements

† The molecular weights of the free bases are practically the same, allowing direct comparisons of the data.

at pH 6.75 but two other maxima are found; in the case of pyridoxine these are at 254 and 325  $m\mu$ . Pyridoxal shows absorption maxima at 251 and 316  $m\mu$ , while pyridoxamine has maxima at 250 and 325  $m\mu$ , the absorption curve in the latter region closely duplicating that of pyridoxine. At the



more alkaline pH of 10.2, the compounds show increased light absorption at the shorter wave-length and decreased absorption in the near ultraviolet. The absorption bands in all cases exhibit shifts toward the shorter wave-length. Thus, the alkaline solutions of both pyridoxine and pyridoxamine show absorption maxima at 246 and 311  $m\mu$ , while for pyridoxal the maxima are at 237 and 301  $m\mu$ . The absorption data demonstrate that both pyridoxal and pyridoxamine show features of amphoterism similar to those exhibited by pyridoxine (14, 16, 17).

In Table I are presented ultraviolet absorption data for pyridoxine, pyridoxamine, and pyridoxal before and after the compounds were subjected to the hydrolytic procedures described above. Readings of the solutions buffered at pH 6.75 were taken only in the range of 300 to 350  $m\mu$ . This portion of the absorption curve has been shown to be responsive to pyridoxine destruction in studies on the stability of the vitamin to light (18). In Table I are listed the extinction coefficients ( $E_1^{1\%}$ ) for the free bases at their respective absorption maxima and the significant extinction ratios, which define the shape of the absorption curves.

The results show that heating pyridoxine and related compounds in acid solutions produces small but appreciable increases in the extinction coefficients. There also occurs a "flaring out" of the absorption curves. These changes are somewhat greater when the compounds are treated with the stronger acid solutions.

*Chemical Studies*—In Table II are presented the results of chemical analyses of the vitamin solutions before and after hydrolysis. The colorimetric method (7) was used with and without the borate blank. The results are expressed in terms of the free bases.

It will be noted that by the published procedure pyridoxamine is about 31 per cent as reactive as pyridoxine on an equimolar basis, whereas pyridoxal is even less, about 16 per cent. In the absence of the borate, both derivatives are about half as chromogenic as pyridoxine. No measurable destruction of any of these compounds occurs as a result of the heat treatments.

Inasmuch as the modifications in the pyridoxine formula are in the 4-hydroxymethyl group, it would seem that no coupling should occur between borate and pure pyridoxal or pyridoxamine (16). Conclusive evidence has been presented for the purity of the pyridoxine derivatives (5). It appears, therefore, that pyridoxal exists in solution as the internal hemiacetal or as the hydrate, thereby furnishing a hydroxyl group adjacent to the phenolic group. Such a relationship has been shown (16) to be required for the formation of the borate complex. In the case of pyridoxamine the partial coupling with borate undoubtedly occurs through the amino group (19). The formation of analogous cyclic complexes between  $\alpha$ -alkanolamines and

lead tetraacetate is favored when the hydroxyl and amine groups are on adjacent carbons (20).

In Table III are shown the results of experiments demonstrating an adaptation of the colorimetric procedure to the specific determination of pyridoxine in the presence of pyridoxal and pyridoxamine. The changes from the published procedure consisted in reducing the ammonia solution in the buffer from 160 to 40 ml. per liter and extending the reaction time from 1 to 15 minutes. The data indicate that pyridoxal and pyridoxamine

TABLE II

*Relative Values Obtained by Colorimetric Procedure for Pyridoxine and Related Compounds Subjected to Various Hydrolytic Procedures\**

Compound	Hydrolytic procedure	Results	
		Published procedure	Procedure without borate blank
Pyridoxine	None	1.00	1.00
	0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min.	0.99	0.99
	4 N HCl, 100°, 60 min.	1.04	1.04
	2 " H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 30 min.	0.94	0.94
Pyridoxamine	None	0.30	0.48
	0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min.	0.31	0.47
	4 N HCl, 100°, 60 min.	0.30	0.47
	2 " H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 30 min.	0.31	0.53
Pyridoxal	None	0.16	0.43
	0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min.	0.13	0.48
	4 N HCl, 100°, 60 min.	0.18	0.50
	2 " H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 30 min.	0.18	0.51

\* Comparisons are made against the chromogenic activity of untreated pyridoxine set at unity. The molecular weights of the free bases are practically the same.

react with the chloroinide reagent to yield a blue pigment but, unlike pyridoxine, the reactions are not inhibited by borate. When all three compounds are present, the total photometric density of blue pigment formed is equal to the sum of the individual photometric densities. The difference in the photometric density, in the absence and presence of borate, is due solely to the contribution of pyridoxine to the reaction.

The application of the colorimetric procedure to three samples of yeast powder is also illustrated in Table III. Yeast has been reported to contain little pyridoxine as such (7, 8), and this is substantiated by the small decreases in the color intensity occurring as a result of the added borate. Tests of aliquots of the solutions, to which known increments of pyridoxine have been added (internal standard procedure), indicate that the small

values for pyridoxine naturally present are not due to interference in the color development; the increase in photometric density due to the added 10  $\gamma$  of pyridoxine approximates the value for the reaction of the vitamin in pure solution.<sup>1</sup>

The values in Table III are based on readings 15 minutes after addition of the chloroimide reagent. However, readings were actually taken at

TABLE III

*Adaptation of Colorimetric Procedure for Determination of Pyridoxine in Presence of Pyridoxal and Pyridoxamine*

Test extract per tube	Photometric density				Pyridoxine hydrochloride equivalent
	Borate blank*	Test†	Test minus blank	Increment per 10 $\gamma$ pyridoxine hydrochloride	
					$\gamma$
(a) 5 $\gamma$ † pyridoxal hydrochloride	0.257	0.266	0.009		0.4
(b) 5.86 $\gamma$ † pyridoxamine dihydrochloride	0.066	0.067	0.001		0.0
(c) 10 $\gamma$ pyridoxine hydrochloride	0.000	0.214	0.214	0.214	10.0
(d) 20 " " "	0.010	0.429	0.419	0.210	20.0
(e) a + b	0.308	0.322	0.014		0.7
(f) " + " + c	0.320	0.548	0.228	0.228	10.7
(g) " + " + d	0.332	0.759	0.427	0.214	20.2
(h) Test solution of Yeast Powder A	0.249	0.260	0.011		0.6
(i) h + 10 $\gamma$ pyridoxine hydrochloride	0.258	0.460	0.202	0.200	
(j) Test solution of Yeast Powder B	0.267	0.302	0.035		1.8
(k) j + 10 $\gamma$ pyridoxine hydrochloride	0.277	0.497	0.220	0.195	
(l) Test solution of Yeast Powder C	0.238	0.250	0.012		0.6
(m) l + 10 $\gamma$ pyridoxine hydrochloride	0.246	0.449	0.203	0.199	

\* For these readings, the colorimeter was set at 100 per cent transmission with a tube containing 5 ml. of isopropanol, 1 ml. of water, 2 ml. of ammonia-ammonium chloride solution, and 1 ml. of boric acid, 15 minutes after the addition of 1 ml. of the chloroimide reagent.

† The colorimeter was set at 100 per cent transmission with a tube containing the same solution as above, except that water was substituted for the boric acid.

‡ Molar equivalents of 5  $\gamma$  of pyridoxine.

1 minute intervals in order to plot the course of the reactions. It was apparent from the data that during the first 10 minutes some borate coupling occurs with pyridoxamine and pyridoxal. Although these reactions are rapid, they appear to be more easily reversed at the lower pH selected for the colorimetric reaction. Thus, after a suitable period, *viz.* 15 minutes, the reaction of pyridoxal and pyridoxamine with chloroimide causes complete dissociation of their borate complexes. In the case of pyridoxine no appreciable dissociation of the borate complex occurs.

**Microbiological Studies**—The results of microbiological assays of solutions of pyridoxine and the related compounds, before and after hydrolysis, are presented in Table IV. Two species of yeast were employed as the test microorganisms, *Saccharomyces cerevisiae* (21, 11) and *S. carlsbergensis* (10). All tubes were incubated at 30° for 18 hours and then read turbidimetrically. Like the colorimetric data, values are reported in terms of free bases, and comparisons are made with the potency of untreated pyridoxine set at unity. The results indicate that none of the compounds is destroyed as a

TABLE IV  
*Relative Microbiological Activity of Pyridoxine and Related Compounds Subjected to Various Hydrolytic Procedures\**

Compound	Hydrolytic procedure	Results with test organism	
		<i>Saccharomyces cerevisiae</i>	<i>Saccharomyces carlsbergensis</i>
Pyridoxine	None	1.00	1.00
	0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min.	0.95	0.95
	4 N HCl, 100°, 60 min.	1.01	0.99
	2 " H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 30 min.	1.02	1.00
Pyridoxamine	None	0.38	0.89
	0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min.	0.37	0.85
	4 N HCl, 100°, 60 min.	0.44	0.86
	2 " H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 30 min.	0.40	0.83
Pyridoxal	None	0.44	0.97
	0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min.	0.48	0.91
	4 N HCl, 100°, 60 min.	0.45	0.90
	2 " H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 30 min.	0.45	1.01

\* Comparisons are made against the potency of untreated pyridoxine set at unity. The molecular weights of the free bases are practically the same.

result of the heat treatments. With *S. carlsbergensis* the three compounds show comparable activity. However, with *S. cerevisiae* pyridoxamine exhibits only 40 per cent of the activity of pyridoxine, while pyridoxal has 46 per cent activity.

The microbiological data, as well as the chemical and spectrophotometric results, indicate that pyridoxine, pyridoxal, and pyridoxamine are not destroyed by any of the hydrolytic procedures employed. However, in the preparation of test extracts for assay, it is essential that an appropriate hydrolytic method be employed, since no general procedure can be suggested at the present time. This conclusion is based upon microbiological (10) and multiple level rat (22) assays conducted on various natural products. Illustrative data are presented in Table V. In the case of the rice

bran concentrate, with increasing concentration of acid increasing pyridoxine values were obtained, the maximum value agreeing with the biological estimate.<sup>8</sup> This has been demonstrated to be due simply to the liberation of bound pyridoxine (12, 11, 7); rice bran contains insignificant quantities of the other members of the B<sub>6</sub> complex (7, 8). On the other hand, when the more effective hydrolytic procedures are employed in the assay of yeast, progressively smaller values for vitamin B<sub>6</sub> content are obtained. Only the value obtained by the weakest of the hydrolytic procedures approximates the bioassay estimate. This would indicate the presence in yeast of a factor which exhibits vitamin B<sub>6</sub> activity but is destroyed by strong acid hydrolysis.

TABLE V

*Variations in Vitamin B<sub>6</sub> Content\* of Biological Materials Dependent upon Selection of Hydrolytic Procedure*

Hydrolytic procedure	Microbiological† values on	
	Rice bran concentrate	Yeast A
	$\gamma$ per gm	$\gamma$ per gm
0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min. . . . .	28	25
0.44 " " 15 " " 90 " . . . . .	95	15
2.0 " " 15 " " 30 " . . . . .	115	10
4.0 " HCl, 100°, 60 min. . . . .	112	8
Rat assay values‡		
None . . . . .	110	28

\* Values expressed as pyridoxine hydrochloride.

† Obtained by using *Saccharomyces carlsbergensis* as the test organism (10).

‡ Conducted according to the method of Dimick and Schreffler (22) in which both growth response and degree of cure of the rat dermatitis are taken into consideration.

The data in Table VI demonstrate that the labile vitamin B<sub>6</sub> factor in yeast is not pyridoxal or pyridoxamine. Theoretical recoveries of the added pyridoxine derivatives, *i.e.* within the precision of the microbiological assay, are obtained regardless of which hydrolytic procedure is employed in the preliminary extraction of the yeast powders.

The results of differential assays on three yeast samples are presented in Table VII. The chemical method was the present adaptation of the colorimetric procedure (7). For measuring the total vitamin B<sub>6</sub> activity of the hydrolyzed sample the yeast (*Saccharomyces carlsbergensis*) growth

\* Increasing further the concentration of the acid employed for the hydrolysis gave the same value for pyridoxine.

method of Atkin and associates (10) was employed. The microbiological assays with *Streptococcus faecalis* R and *Lactobacillus casei* were conducted<sup>4</sup> according to the methods described by Snell and Rannefeld (9) involving the aseptic addition of the hydrolyzed samples to previously sterilized media (8). Pyridoxine is relatively inactive for both lactic acid bacteria; pyridoxamine exhibits a negligible stimulatory effect on *Lactobacillus casei*, while pyridoxal is effective; both pyridoxamine and pyridoxal are active for *Streptococcus faecalis* (8), the latter compound to a lesser extent, exhibiting about 36 per cent of the activity of pyridoxamine.

TABLE VI

*Evidence for Presence of Other Factors in Vitamin B<sub>6</sub> Complex in Brewers' Yeast\**

Sample	Microbiological† values on hydrolyzed samples	
	2 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs pressure, 30 min.	0.055 N H <sub>2</sub> SO <sub>4</sub> , 15 lbs. pressure, 90 min
	$\gamma$ per gm	$\gamma$ per gm
A	10	25
B	23	38
1.00 gm. Sample A + 34 $\gamma$ pyridoxine·HCl	43	59
1.00 " " " + 20 " pyridoxal·HCl + 20 $\gamma$ pyridoxamine·2HCl‡	38	59
1.00 gm. Sample B + 34 $\gamma$ pyridoxine·HCl	57	70
1.00 " " " + 20 " pyridoxal·HCl + 20 $\gamma$ pyridoxamine·2HCl‡	62	72

\* All values are expressed as pyridoxine hydrochloride.

† Obtained by using *Saccharomyces carlsbergensis* as the test organism (10).

‡ The combined increment of pyridoxal hydrochloride and pyridoxamine dihydrochloride should be equivalent in microbiological response to 34  $\gamma$  of pyridoxine hydrochloride.

The data summarized in Table VII indicate that only a small fraction of the vitamin B<sub>6</sub> in yeast is pyridoxine. The microbiological values obtained only with the acid-hydrolyzed (2 N H<sub>2</sub>SO<sub>4</sub>) Sample B are confirmatory of those reported by Snell (8) for yeast. In the case of the other two samples (also hydrolyzed with 2 N H<sub>2</sub>SO<sub>4</sub>), the vitamin B<sub>6</sub> activities as estimated from the assays with the lactic acid bacteria are far in excess of the esti-

<sup>4</sup> The folic acid preparations added to the basal medium were vitamin B<sub>c</sub> (23) and Stokstad's crystalline material isolated from liver (24). We are indebted to Dr. O. D. Bird, Parke, Davis and Company, Detroit, for furnishing us with the former compound and to Dr. E. L. R. Stokstad, the Lederle Laboratories, Inc., Pearl River, for the latter material.

mates derived from the yeast growth assays. This seemingly paradoxical finding is comparable to the results obtained by Snell (8) in assays of liver extract. When the tests are conducted on samples prepared by 0.055 N H<sub>2</sub>SO<sub>4</sub> hydrolysis, greatly increased microbiological activity for the lactic acid bacteria is observed over that noted in the yeast growth assays. The known activity of pyridoxine, pyridoxal, and pyridoxamine for the test microorganisms and the stability of these compounds exclude their responsibility for the enhanced microbiological growth.<sup>5</sup> The labile factor in the vitamin B<sub>6</sub> complex, however, behaves like the pyridoxine derivatives in that it exhibits activity comparable to pyridoxine in yeast and rat growth assays. Consequently, the validity of the differential microbiological

TABLE VII

*Differential Assays\* of Yeast Samples for Vitamin B<sub>6</sub> after Autoclave Extraction with 2 N and 0.055 N Sulfuric Acid*

Sample	Chemical† procedure	<i>Saccharomyces carlsbergensis</i> ‡		<i>Streptococcus faecalis</i> §		<i>Lactobacillus casei</i> ¶	
		2 N H <sub>2</sub> SO <sub>4</sub>	0.055 N H <sub>2</sub> SO <sub>4</sub>	2 N H <sub>2</sub> SO <sub>4</sub>	0.055 N H <sub>2</sub> SO <sub>4</sub>	2 N H <sub>2</sub> SO <sub>4</sub>	0.055 N H <sub>2</sub> SO <sub>4</sub>
A	3	10	25	17	58	1.9	10
B	11	23	38	20	55	2.8	14
C	2	7	17	12	24	1.6	11

\* All values are expressed as micrograms per gm. of pyridoxine hydrochloride on an equimolar basis.

† Pyridoxine hydrochloride standard; present adaptation (see Table III) of colorimetric method (7) to the specific measurement of pyridoxine.

‡ Pyridoxine hydrochloride standard.

§ Pyridoxamine dihydrochloride standard.

¶ Pyridoxal hydrochloride standard.

assay technique (8) for fractionating the vitamin B<sub>6</sub> group is open to question, since it is predicated upon the assumption that the sole members of the complex are pyridoxine, pyridoxal, and pyridoxamine. However, this does not detract from the importance of the differential assays in demonstrating (8) the presence of pyridoxal and pyridoxamine in biological materials.

<sup>5</sup> Thymine can replace folic acid in the *Lactobacillus casei* and *Streptococcus faecalis* assays (25, 26). Since this compound is not included in the present basal medium, tests were conducted in order to determine whether the high values obtained by the lactic acid bacteria assays might be due to the presence of the pyrimidine. It was found that this compound could not replace the pyridoxine derivatives. The thymine was kindly supplied by Dr. J. L. Stokes, Merck and Company, Inc., Rahway.

## DISCUSSION

The spectrophotometric, chemical, and microbiological data reported in this paper demonstrate that pyridoxine, pyridoxamine, and pyridoxal are all stable to the various hydrolytic procedures employed in preparing biological materials for assay. Cunningham and Snell (27) have recently found the derivatives to be as stable as pyridoxine when subjected to the same series of tests employed in studies with the latter compound (18). In the present study small increases were noted in the extinction coefficients ( $E_{1\text{ cm.}}^{1\%}$ ) at the respective absorption maxima and slight changes in the absorption curves of the acid-treated compounds; these are regarded as of no significance, since they are not reflected in either the chemical or microbiological assays.

The ultraviolet absorption curves show no maxima common to all three compounds. However, if readings are taken at 325  $m\mu$  of solutions at pH 6.75, close estimates of the total amount of the compounds present may be obtained, despite variations in their relative concentrations. At that wavelength there is an absorption maximum common to pyridoxine and pyridoxamine, and, while pyridoxal absorbs approximately 20 per cent more light at its 315  $m\mu$  maximum, at 325  $m\mu$  all three compounds absorb to the same extent. The  $E_{1\text{ cm.}}^{1\%}$  values at 325  $m\mu$  in pure solution at pH 6.75 all approximate  $440 \pm 1.5$  per cent, expressed in terms of the free bases. Spectrophotometric analyses, of course, cannot be applied unless the test solutions are free from irrelevant light-absorbing materials, or unless blank solutions containing none of these vitamin B<sub>6</sub> factors are available for evaluating the interference.

By the chemical method as originally published (7) pyridoxamine and pyridoxal are measured to a small extent. As here described, it is readily adaptable to the specific measurement of pyridoxine in pure systems containing varying proportions of pyridoxal and pyridoxamine. Nevertheless, precautions must be exercised in interpreting the results obtained in testing biological materials specifically for pyridoxine content, since a difference method is involved. Only when a major proportion of the total vitamin B<sub>6</sub> activity is due to pyridoxine can precise values be obtained. Metabolically active tissues, such as yeast, contain only small amounts of pyridoxine (8), so that results obtained by chemical assays of these materials can be regarded only as approximations.

The objections to the earlier chemical procedures with phenol reagents (28, 29) have been presented (7). Despite these criticisms and the fact that papers have appeared demonstrating the multiple nature of vitamin B<sub>6</sub> (6, 7), another such non-specific procedure (30) has just been published which fails to take into account errors inherent in the assay technique and



the progress made in the fractionation of the vitamin B<sub>6</sub> group. Pyridoxine and vitamin B<sub>6</sub> are not synonymous (6, 7).

Microbiological methods based on the use of *Saccharomyces cerevisiae* (21, 11), even after provisions are made for the hydrolysis of the bound forms of vitamin B<sub>6</sub> (11), give minimum values for the over-all vitamin B<sub>6</sub> activity, since pyridoxal and pyridoxamine are both less active than pyridoxine for this species of yeast. These results are confirmatory of those reported by Snell and Rannefeld (9) and explain our previous observations (7) that microbiological assays with *Saccharomyces cerevisiae* tend to approach but never exceed the bioassay estimates.

Of the methods here studied for the determination of vitamin B<sub>6</sub> in biological materials, the most reliable is the microbiological procedure of Atkin and associates (10) with *Saccharomyces carlsbergensis*. All members of the complex, those known and those still unidentified, show activity comparable to that of pyridoxine for both this organism and the rat. However, greater emphasis must be directed toward the proper preparation of the sample, *i.e.* for the hydrolysis of bound vitamin B<sub>6</sub>, before a reliable value can be obtained. This point has been largely neglected despite its mention by Atkin and collaborators (10) in describing the details of their procedure. Some materials must be subjected to strong acid hydrolysis such as autoclaving in 2.0 N sulfuric acid solution (11), while others require mild hydrolytic procedures such as 0.055 N sulfuric acid (10). Enzymic digestion procedures likewise must be critically evaluated. Clarase digestion has been demonstrated to be effective in liberating bound vitamin B<sub>6</sub> in yeast (10) but unsatisfactory in testing rice bran (7). The lower values for vitamin B<sub>6</sub>, following the strong acid hydrolysis of some samples, are not due to the destruction of pyridoxine, pyridoxamine, or pyridoxal but of an unidentified labile factor. That this compound should be included in measurements of vitamin B<sub>6</sub> activity is borne out by the observation that it possesses activity (expressed in terms of pyridoxine) for the rat comparable to that for *Saccharomyces carlsbergensis*. However, it is considerably more active than the pyridoxine derivatives, pyridoxal and pyridoxamine, for the lactic acid bacteria. Because of the presence of this factor in biological materials and because it can be responsible for exaggerated microbiological growth when the lactic acid bacteria are employed for assay purposes, the differential microbiological assay technique (8) can lead to erroneous conclusions when employed in fractionating the vitamin B<sub>6</sub> group.

The one disadvantage in the microbiological assay procedure with *Saccharomyces carlsbergensis* is its somewhat low degree of precision. On the average, values may be regarded to be reproducible to within 10 per cent, but frequently larger deviations are encountered of the order of magnitude of 15 per cent. The precision of the chemical procedure (7) is a definite

asset in the analysis of pharmaceutical products which are known to contain only pyridoxine. The method is rapid, simple, and reproducible to within 2 per cent. Inasmuch as pyridoxine is the sole member of the vitamin B<sub>6</sub> complex added in such preparations, the chemical method can be used as originally published (7). The modification described in this report is not as precise or as sensitive.

#### SUMMARY

Ultraviolet absorption curves from 220 to 350 m $\mu$  are presented for pure solutions of pyridoxine, pyridoxamine, and pyridoxal at widely separated pH values. These indicate that pyridoxal and pyridoxamine show features of amphoterism similar to those exhibited by pyridoxine. A spectrophotometric procedure is described for testing pure solutions in which the three compounds vary in relative concentration to each other.

An adaptation of the colorimetric procedure (simply by reducing the pH of the reaction and extending the reaction time) allows specific determination of pyridoxine in the presence of the derivatives. Pyridoxine couples with borate and is thereby rendered non-reactive, whereas pyridoxal and pyridoxamine still react with the chloroimide reagent.

Microbiological assays with *Saccharomyces cerevisiae* underestimate vitamin B<sub>6</sub> content because the biologically active pyridoxine derivatives are appreciably less stimulatory for this organism. These compounds, however, show comparable activity for *Saccharomyces carlsbergensis* and for the rat.

The spectrophotometric, chemical, and microbiological data demonstrate that pyridoxine, pyridoxal, and pyridoxamine are all stable to the various hydrolytic procedures employed for the preparation of samples for assay.

Evidence is brought forth for the presence of an unidentified labile factor in yeast which possesses comparable vitamin B<sub>6</sub> activity for the rat and for *Saccharomyces carlsbergensis*. Because of the natural occurrence of this factor and because some materials require heating in strong acid solutions for liberating bound vitamin B<sub>6</sub>, each type of product should be critically studied for selection of an appropriate method for hydrolysis.

The most reliable of the procedures studied for the determination of vitamin B<sub>6</sub> in natural materials is the microbiological method with *Saccharomyces carlsbergensis*. However, for the assay of pharmaceutical preparations in which pyridoxine is the only member of the vitamin B<sub>6</sub> complex present, the simplicity, rapidity, and precision of the chemical procedure (as originally published) make it the method of choice.

The limitations of the differential microbiological assay technique for fractionating the vitamin B<sub>6</sub> group, depending upon the responses obtained in assaying biological materials with *Saccharomyces carlsbergensis*, *Strepto-*

*coccus faecalis*, and *Lactobacillus casei*, are apparent, since the procedure is based upon the assumption that the sole members of the complex are pyridoxine, pyridoxal, and pyridoxamine.

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## FRACTIONATION OF HOG ADRENAL CORTX EXTRACT

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In a previous report from this laboratory (1) the preparation and comparative physiologic activities of beef, hog, and sheep adrenal cortex extracts were described. By both the rat survival-growth test (2) and the work performance<sup>1</sup> test (3) it was found that the hog adrenal extract was considerably more potent than either beef or sheep adrenal extracts. Because of the high activity by the work test, which is believed to be specific for the assay of the 11-oxygenated steroids that affect carbohydrate metabolism, it was concluded that the higher activity was due to increased amounts of 11-oxygenated steroids in the hog extract. This comparatively high activity has subsequently been confirmed by Olson *et al.* (4), who found a hog adrenal extract to be considerably more potent than several beef adrenal extracts in stimulating glycogen deposition in fasted adrenalectomized rats.

This paper is concerned with the further fractionation studies carried out with the hog adrenal cortex concentrates. The results substantiate our earlier conclusions, based on the data obtained with the work performance test, that the increased activity of these extracts is due principally to a much higher concentration of the 11-hydroxylated steroids. The crystalline adrenal cortex hormones, which are most active in influencing carbohydrate metabolism, can be obtained in considerably greater quantity from hog than from beef adrenal tissue.

By fractionation between benzene and water (5) the more water-soluble  $C_{21}O_6$  compounds were separated from the  $C_{21}O_4$  compounds which remain in the benzene. By exhaustive crystallization of the more water-soluble fractions only biologically active  $C_{21}O_6$  compounds were obtained. By removal of these  $O_6$  compounds the activity by the work test was reduced, whereas by the survival-growth test it was increased. There is thus obtained from hog gland a biologically highly active non-crystalline fraction, the isolation of which has previously been described from beef gland (6-8).

### EXPERIMENTAL

As starting material for this investigation the fat-free biologically active fraction previously described was used. The acidic and basic materials

<sup>1</sup>1 unit is the equivalent in biological activity of 0.2 mg. of 11-dehydro-17-hydroxycorticosterone.

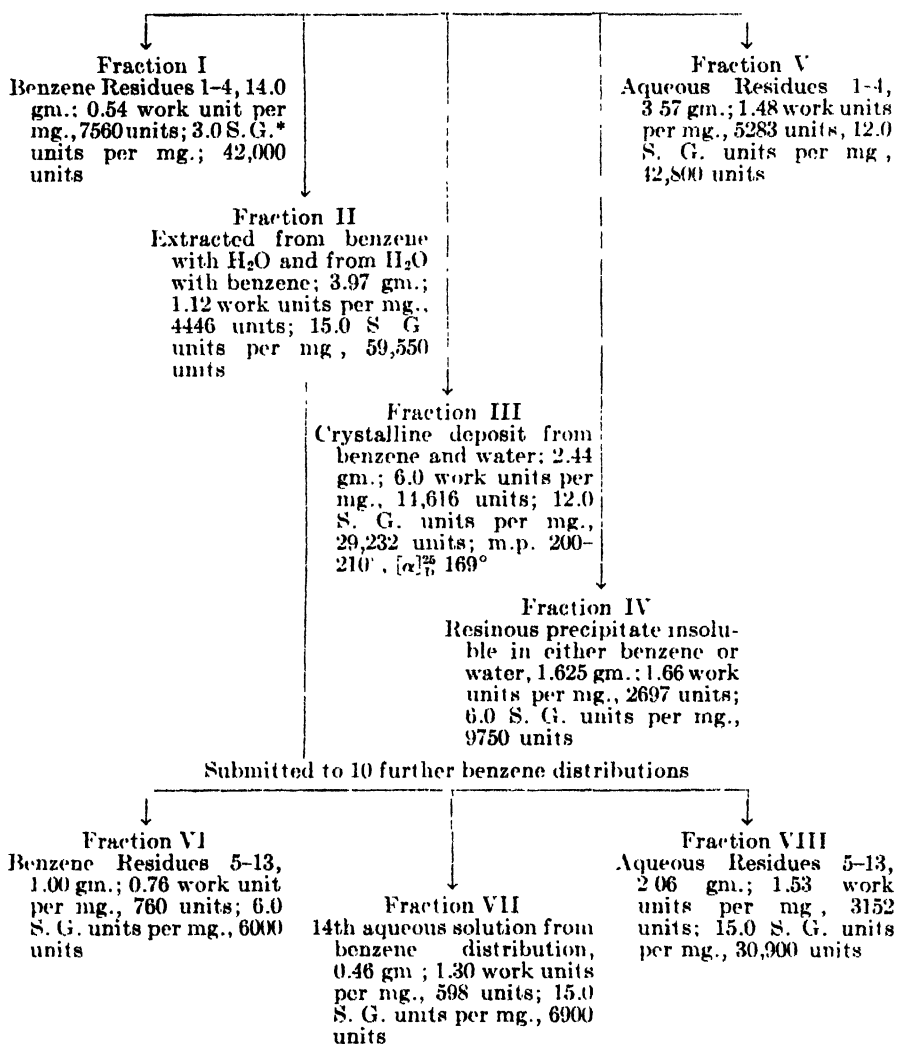
were removed without loss of activity (1). 25.88 gm., obtained from 900 kilos of hog adrenal glands, were fractionated between benzene and water. The ethyl acetate solution was concentrated *in vacuo* below 50° and the residue was dissolved in 300 cc. of methanol. 500 cc. of water were added and an aqueous suspension of the biologically active material was obtained by removing the methanol *in vacuo*. This aqueous residue of 350 cc. was extracted with eight 400 cc. portions of benzene. A resinous precipitate was obtained by concentration of the benzene solution *in vacuo* to 400 cc. This solution plus the precipitate was extracted with eight 400 cc. portions of water at 40°. The material insoluble in benzene and water was separated, finally, by decanting and the aqueous solution of about 3 liters was concentrated *in vacuo* to 400 cc., whereupon a crystalline deposit was obtained. This was removed by filtration and the water solution again extracted with eight 400 cc. portions of benzene. Concentration of the benzene again yielded a small quantity of resin. The benzene concentrate plus resin was treated as above and this procedure continued until, after the fourth distribution, no more crystalline or resinous precipitates were obtained by concentration of either benzene or water. The five fractions thus obtained were assayed and the results are given in the accompanying diagram. By the work test 34,600 units, or 81 per cent of the total activity in the starting material, were recovered in the five fractions. As measured by the survival-growth test, the total amount of activity recovered is practically the same; namely, 82 per cent.

Fraction II is that material which was extracted from benzene with water and from water with benzene. To test whether a biologically more highly active fraction could be prepared, ten more benzene distributions were carried out and Fractions VI, VII, and VIII were obtained. As is seen from the diagram, 1.0 gm. of material remained in the benzene after extraction with water. There was, however, no appreciable further concentration of activity in the intermediate fraction. All the activity by the work test was found in Fractions VI to VIII, obtained from Fraction II, whereas 74 per cent of the activity was recovered by the survival-growth test.

Because of a tendency toward increased nitrogen concentration in the last three aqueous residues of Fraction VIII, these were kept separate. The remaining 1.74 gm. of this most water-soluble material were crystallized from chloroform and yielded 0.150 gm. of crystalline material. Fractions IV and V were likewise crystallized from chloroform and yielded respectively 0.375 and 0.508 gm. of crystals. These crystalline deposits from chloroform were combined with Fraction III, since according to solubility, melting point, specific rotation, and bioassay they were very similar, as can be seen from Table I. The assay of these fractions by the work test indicates that they are a mixture of 11-dehydro-17-hydroxycorti-

*Fractionation of Hog Adrenal Extract by Distribution between Benzene and Water*

Neutral ethyl acetate fraction (25.88 gm.), 1.63 work units per mg., total 42,370; 86 survival-growth units per mg., total 223,000. Submitted to 4 distributions between benzene and water



\* The term "survival-growth" is designated by S. G.

costerone and 17-hydroxycorticosterone, since assay values between those for these two compounds were obtained. These substances were obtained in pure form by following the specific rotation in 95 per cent alcohol. Melting point determinations are of no value as a criterion for separation, since

they are practically identical and show little if any depression when the compounds are mixed. The specific rotations of pure samples of these compounds previously isolated (8) were found to be  $164^\circ$  for 17-hydroxycorticosterone and  $195^\circ$  for 11-dehydro-17-hydroxycorticosterone. These values are in agreement with those reported in the literature (6, 9). By fractional crystallization from acetone it was found that the material least soluble in this solvent could be obtained in pure form, since on repeated crystallization no difference in specific rotation could be effected. 1.2 gm. of this substance were obtained, which, as is evident from the data in Table I, is undoubtedly 17-hydroxycorticosterone. A mixed melting point with a pure sample of this compound previously obtained (8) gave no depression.

TABLE I  
*Chloroform-Insoluble Fractions from Hog Adrenal Extract*

Preparation	Weight	M.p., corrected	[ $\alpha$ ] <sub>D</sub> <sup>25</sup> in 95 per cent EtOH	Bioassay	
				Work	Survival- growth
	gm.	°C.		units per mg.	units per mg.
Fraction III, CHCl <sub>3</sub> -insoluble ..	2.44	200-210	169	6.0	12.0
CHCl <sub>3</sub> -insoluble of Fraction IV...	0.43	196-208	155	6.2	
"      "      "      VIII.	0.51	202-210	162		
Acetone recrystallization of CHCl <sub>3</sub> - insoluble ..	1.2	215-218	164	7.3	10.0
EtOH recrystallization of acetone mother liquors . . .	0.68	212-215	200	5.7	10.0

The acetone mother liquors were combined and crystallized from 95 per cent ethanol. By recrystallization from this solvent the specific rotation could be increased until a value of  $200^\circ$  was attained, after which further crystallization did not increase the rotation. Beautiful rhombohedra weighing 0.68 gm. were obtained. The bioassay result by the work test taken with the physical data identifies this material as 11-dehydro-17-hydroxycorticosterone, a pure sample of which gave no melting point depression with the crystals here obtained.

The chloroform-insoluble material of Fraction V was obtained after a preliminary purification by removal of acidic and basic material. The weight of the fraction was thus cut down from 3.57 to 2.21 gm. By crystallization from chloroform 0.51 gm. assaying 6.5 units per mg. were removed. This represents a removal of 3315 work units and about 5100 survival-growth units. According to the bioassay results, the removal of

the relatively large amount of 1.36 gm. of acidic and basic material resulted in no loss of activity by the work test, since the 1.7 gm. assayed 1.06 units per mg. 1800 of a possible 1900 units were, therefore, recovered. By the survival-growth test 25,600 units out of a possible 37,700 were recovered.

The 1.7 gm. of Fraction V after removal of acidic and basic material and  $\text{CHCl}_3$ -insoluble crystals were separated into ketonic and non-ketonic fractions with Girard's reagent. The material was dissolved in 21 cc. of absolute methanol and 2 gm. of Girard's reagent, and 1.04 cc. of glacial acetic acid were added. This mixture was kept at room temperature overnight and was then cooled to  $-10^\circ$ . With constant shaking 26.0 cc. of normal  $\text{Na}_2\text{CO}_3$  solution were added and, with the receiver in an acetone-dry ice mixture, the methanol was removed at  $0-5^\circ$ . The resulting aqueous solution was extracted with seven 100 cc. portions of ethyl acetate. The combined ethyl acetate solution of the non-ketonic material was washed back with 50 cc. of water. The aqueous solution plus the washing of the ethyl acetate was acidified by adding 0.1 volume (10 cc.) of concentrated  $\text{HCl}$  and the ketonic material was extracted with eight 100 cc. portions of ethyl acetate. The ethyl acetate solution was washed with 1 per cent  $\text{Na}_2\text{CO}_3$  followed by  $\text{H}_2\text{O}$ , until the washings were no longer alkaline. The non-ketonic fraction weighed 0.59 gm. and the ketonic fraction 1.09 gm. A quantitative recovery of activity by the work test was obtained in the ketonic fraction, since this assayed 1.7 units per mg. and, therefore, contained 1853 units. More than half of the activity by the survival-growth test was lost, since the ketonic fraction assayed only 10 units per mg., and no activity could be discerned in the non-ketonic fraction at 10 times this dose.

Only 30 mg. of material could be obtained from the ketonic fraction by crystallization from chloroform. According to specific rotation and melting point this was 17-hydroxycorticosterone. The non-crystallizable 1.05 gm. were acetylated with acetic anhydride in pyridine, and the neutral fraction from the acetylation weighed 0.95 gm. This material was fractionated by being placed on an aluminum oxide column and eluted with various solvents. 10 gm. of Merck's (Darmstadt) aluminum oxide, standardized according to Brockmann, were used. It was washed with petroleum ether and the fraction to be adsorbed was dissolved in 75 cc. of pure anhydrous benzene. 25 cc. of pure hexane were added and the clear solution run through the column. The filtrate contained no solids. The material was then fractionally eluted by perfusing with benzene, benzene and ether, ether, ether and acetone, acetone, and methyl alcohol, the procedure being practically the same as that used by von Fuw and Reichstein (10) for fractionation of the more water-soluble concentrates of beef



adrenal glands. After the material was rechromatographed two times, a high melting acetate was obtained in the first fractions eluted with benzene. Only 13 mg. of this substance which melted at 260–267° (corrected) could be obtained. Due to lack of material it has not been investigated further. The fractions eluted with benzene and ether mixtures containing 2 to 6 per cent ether yielded 45 mg. of a pure acetate melting at 220–222° (corrected). A mixed melting point with an authentic sample of 17-hydroxycorticosterone acetate showed no depression. All the other fractions obtained from the column were amorphous. Only about 75 per cent of the substances could be eluted from the column, and this was found by the work test to be less than one-tenth as active as it was before placing it on the aluminum oxide column. For this reason chromatographic separation of this material on aluminum oxide was not investigated further.

A nitrogen analysis by the Dumas method on Fraction VII gave a value of 4.6 per cent. This is surprisingly high and indicates that a nitrogen-containing substance is concentrated in this fraction, since the nitrogen content of the original neutral ethyl acetate fraction (*cf.* the diagram) before benzene distribution is less than 1.0 per cent.

Bioassay data on Fractions VII and VIII are very similar. Nitrogen determinations were run and it was found that Aqueous Residues 11, 12, and 13 had practically the same high nitrogen content as the last material extracted from the water with the benzene. A preliminary distribution between ethyl acetate and water resulted in a higher concentration of nitrogen in the aqueous phases. In an attempt to separate the nitrogen-containing substance, the following procedure was, therefore, carried out. Aqueous Residues 11, 12, and 13 weighed 0.324 gm. and were combined with Fraction VII. The 0.78 gm. was dissolved in 100 cc. of ethyl acetate. Five 250 cc. separatory funnels were set up and the solution placed in the first funnel. 100 cc. portions of ethyl acetate were placed in each of the other funnels. The solution was shaken with 50 cc. of water and the water extract was passed in order through the second, third, fourth, and fifth funnels. Ten such water extractions were carried out. The material remaining in the combined ethyl acetate solution weighed 0.49 gm., while in the combined water extracts there was 0.27 gm. which contained 8.89 per cent nitrogen. This material was crystallized from 1 cc. of alcohol and yielded 130 mg., m.p. 150–160°. By sublimation in a vacuum of  $5 \times 10^{-5}$  mm. of Hg at a temperature of 105–110° and crystallization from pure acetone, 68.3 mg. were obtained, which melted at 162–164° (corrected). By both the work and survival-growth assays the substance was found to be inactive and is undoubtedly identical with the nitrogenous Compound E of Wintersteiner and Pfiffner, which these authors isolated from a beef

adrenal concentrate, and believed to be *l*-leucyl-*l*-proline anhydride (11). The following analytical data were obtained.

$C_{11}H_{19}O_2N_2$ . Calculated. C 62.81, H, 8.63, N, 13.33  
 Found. " 63.15, " 8.62, " 13.74  
 " 63.19, " 8.73

The 0.49 gm. from the ethyl acetate solution was dissolved in 3 cc. of acetone, and 27 cc. of water were added. After standing in the refrigerator overnight, 0.131 gm. of insoluble material was removed by centrifuging. The 0.360 gm. which remained in solution contained 0.83 per cent nitrogen. This assayed 20 units per mg. by the survival-growth assay method and 0.9 unit per mg. by the work test. Biological assay data on this fraction

TABLE II

*Biological Activity of Water-Soluble Amorphous Fraction from Hog Adrenal Glands*  
 The results are expressed in units per mg.

Preparation	Survival-growth	Dog assay	Work assay	Corner-Allen progestational test
Desoxycorticosterone	35	500	0.1	0.1
Progesterone	0.25			1.0
Water-soluble amorphous hog adrenal fraction	20	500	0.9	<0.02
Beef adrenal extract, 1 cc = 40 gm. gland	4	110	1.0	<0.02

are given in Table II in which they can be compared with those for desoxycorticosterone and progesterone. The dog assay was run according to the method of Pfiffner *et al.* (12). The value of 500 dog units per mg. of this amorphous fraction is the average obtained when in parallel assays desoxycorticosterone gave a value of 500 and a beef adrenal cortex extract (13) gave a value of 110 units per cc., each cc. being equivalent to 40 gm. of gland. It can be seen that the tests for adrenal cortex activity indicate a great similarity between desoxycorticosterone and the amorphous fraction. The progestational effect of the three substances was, therefore, also determined. Desoxycorticosterone, as has previously been reported (14), has a very pronounced progestational effect. By the Corner-Allen method of assay (15) we found that at a 10 mg. dose it gave a slightly greater response than 1 mg. of pure progesterone. It contains, therefore, about 0.1 Corner-Allen unit per mg. When the adrenal cortex fraction was assayed at a dosage level of 50 mg., no progestational activity whatever could be discerned. By comparing the results by the survival-growth assay and by the pro-

gestational activity test it is apparent that the cortical hormone activity is probably not due to content of desoxycorticosterone.

#### DISCUSSION

Hog adrenal cortex concentrates made by a procedure comparable to that used for beef adrenal extracts are very characteristically different in that the 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone are easily crystallizable from the benzene-insoluble fractions. Similar fractions from beef glands contain such a high percentage of biologically inactive  $C_{21}O_5$  compounds (8) that the crystalline deposits obtained after distribution between benzene and water contain these compounds almost exclusively. In our experience it was necessary to fractionate further into ketonic and non-ketonic fractions in order to obtain the crystalline biologically active  $C_{21}O_5$  compounds. As has previously been pointed out (16), a summary of the yields of biologically active crystalline fractions obtained from beef and hog adrenal extracts shows that a much higher percentage of the total activity of the latter can be recovered in crystalline form. By the work test this accounts for 65 per cent of the composite activity in the neutral ethyl acetate-soluble fraction from hog gland, whereas it accounts for only 33 per cent of the activity in the identical fraction from beef gland. Only about 75 to 100 mg. of 17-hydroxycorticosterone, which is most active by carbohydrate function tests (3, 4), can be obtained per thousand pounds of beef glands. In contrast 600 mg. per thousand pounds of hog glands can quite easily be obtained in the purest form. In these extracts concentration of this compound predominates somewhat over that of 11-dehydro-17-hydroxycorticosterone, of which 340 mg. per thousand pounds were obtained. Examination of the benzene residues has also revealed that only corticosterone and not 11-dehydrocorticosterone could be crystallized. Taken altogether, the evidence indicates that the synthesis of the hormones in the cortex of the hog adrenal gland is predominantly that of the 11-hydroxy rather than the 11-ketosteroids.

After removal of these pure compounds of high activity by the work test, a fraction can be obtained from hog as well as from beef gland which is of low activity by the work test and of high activity by both the survival-growth assay (2) and the Pfiffner-Swingle dog assay (12). Although desoxycorticosterone has a similar type of activity, we do not believe this can be present in this so called "amorphous fraction." The activity of this fraction by oral administration was found to be as great as by injection, whereas desoxycorticosterone administered orally is less than one-twentieth as active as it is parenterally (17). Furthermore, to account for the activity by the survival-growth test this water-soluble fraction would have to

consist of at least half desoxycorticosterone, which does not seem possible since it is very insoluble in water. That such a concentration of desoxycorticosterone cannot exist in this fraction is also shown by the results of the progestational activity test by which 50 mg. gave no response, whereas 10 mg. of desoxycorticosterone give the full progestational proliferation produced by 1 mg. of progesterone. The only substance that could be crystallized from this was the nitrogenous compound believed to be *l*-leucyl-*l*-proline anhydride, which does not seem to bear any relationship to the biological activity. The results of fractionation show that this activity is easily lost by chemical manipulation, since by the growth-survival test only half could be recovered after separation with Girard's reagent, and after adsorption on aluminum oxide apparently all the biological activity was lost.

#### SUMMARY

The relatively high activity of hog adrenal extracts by the work test is due to a high concentration of 11-oxygenated steroids most active by this test. About 6 times as much 17-hydroxycorticosterone, which is the compound most active by carbohydrate function tests, could be crystallized from this source as from an equal amount of beef adrenal extract. After removal of the 11-oxygenated steroids by as complete crystallization as possible, there can be prepared from the extracts of hog as well as beef a water-soluble fraction of relatively low activity by the work test and of high activity by the growth-survival test. This activity of the so called amorphous fraction does not seem to be due to any of the known biologically active adrenal steroids.

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# THE UTILIZATION OF *d*-AMINO ACIDS BY MAN\*

## III. ARGININE

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Although the commercial availability of naturally occurring arginine deprives the present report of practical implications in human nutrition, the investigation seems experimentally noteworthy because not only could the metabolic fate of the ingested *d*(-)-arginine be deduced from measurements of urinary metabolites, but also direct evidence on one phase of the process could be secured from the *in vitro* action of human liver arginase on the unnatural form. Our feeding experiments showed that the oral administration of 0.01 mole of *dl*-arginine hydrochloride (equivalent to 0.87 gm. of *d*(-)-arginine) resulted in the excretion of 100 to 160 mg. more of arginine than the feeding of 0.01 mole of *l*(+)-arginine hydrochloride. The absence of significant qualitative or quantitative differences in the urinary output of other N metabolites, especially amino N, following the ingestion of *l*(+)- and *dl*-arginine was interpreted to indicate that approximately 80 per cent of *d*(-)-arginine fed follows the metabolic path of *l*(+)-arginine. This interpretation received some experimental support from the unexpected finding that the quantitative equivalents of urea were obtained from both *l*(+)- and *dl*-arginine on incubation with crude arginase prepared from human liver. This observation naturally raises questions on the nature of the reaction mechanism involved. Two possibilities which present themselves are (a) that both *d*- and *l*-arginase occur in the human liver, or (b) that *d*(-)-arginine is first biochemically converted to the *l* isomer and then acted upon by *l*-arginase. The single *in vivo* difference, the urinary loss of arginine following the oral administration of the racemate, fails to resolve this ambiguity, since it could be argued with equal validity that this phenomenon is due to a lag in either possible enzyme reaction with the *d* form. The results of the *in vitro* experiments indicate that no differences in reaction rates exist and lead one to suspect that the observed

\* The work described in this report was supported in part by grants from the Rockefeller Foundation and the Nutrition Foundation, Inc. Partial support was also derived under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Johns Hopkins University.

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arginine loss is merely due to a lower kidney threshold value for the unnatural form. However, the use of *in vitro* results for the interpretation of *in vivo* data is obviously questionable. Despite the inadequacy of the data to reveal biochemical details, our observations indicate clearly that unnatural arginine is partially metabolized by man.

Quite apart from the primary objective of the present study, observations on the action of rat liver arginase on *dl*- and *l*(+)-arginine demonstrated that although 1 molecular equivalent of urea was obtained from *l*(+)-arginine, only 0.5 equivalent of urea was obtained from *dl*-arginine. This finding suggests that the *d* isomer may not be utilized by the rat.

#### EXPERIMENTAL

*Preparation of dl-Arginine Hydrochloride*—Commercially available *l*(+)-arginine hydrochloride, Merck, found to contain 26.6 per cent of N and to have a specific rotation of  $[\alpha]_D^{20} = +12.5^\circ$  in 3.4 per cent aqueous solution, was used in all experiments. *dl*-Arginine hydrochloride was prepared by racemization of the *l* form with acetic anhydride by a modification of the procedures of Bergmann and Zervas (1) and du Vigneaud and Meyer (2) as follows: 20 gm. of *l*(+)-arginine hydrochloride, Merck, were dissolved by heating on the steam bath in a mixture of 25 cc. of acetic anhydride and 50 cc. of glacial acetic acid. After storage for 24 hours at 37°, the excess acetic acid was removed by concentrating the reaction mixture *in vacuo* to a thick syrup three times successively, after the addition of water. The final syrup was dissolved in 100 cc. of 6 N HCl and the solution was boiled under a reflux for 3 hours in an all-glass apparatus. The excess of hydrochloric acid of this solution was removed by three evaporations *in vacuo* following additions of water. The final syrup was dissolved in 100 cc. of 95 per cent ethanol and the monohydrochloride was precipitated from solution by the slow addition of a 1:1 mixture of pyridine and ethanol. After 24 hours of refrigeration the crude crystalline substance was collected and purified by dissolving the product in 25 cc. of hot water containing 1 gm. of norit A and precipitation from the hot filtered solution by the gradual addition of 2 volumes of 95 per cent ethanol. The final product weighed 17.0 gm. and was found to be optically inactive and to contain 26.5 per cent N (theory 26.6) by micro-Kjeldahl analysis (3).

*Human Experiments*—As in our previous investigations of this series, three fasting adult subjects were given 2.10 gm. (0.01 mole) of *l*(+)- or *dl*-arginine hydrochloride in 240 cc. of water 2 hours after breakfast and 120 cc. more of water at the end of each hour of the experimental periods to maintain an optimum flow of urine. The duration of the experiments varied from 3 to 24 hours. Since it was found that the intake of food did

not appreciably affect the urinary arginine output, the fast was imposed only for the first 3 hours of the 24 hour periods. The urine which was collected for the 2 hour period prior to administration of arginine is designated as urine collected at zero hour. The urine was collected hourly thereafter for 6 hours or longer and was analyzed for total N (3), arginine (4), amino N (5), urea (6), and creatine and creatinine (7).

Arginine excretion curves of one of five experiments are shown in Fig. 1 and indicate that, whereas the ingestion of *l*(+)-arginine results in only slightly greater output of arginine than when no arginine is fed, the feeding

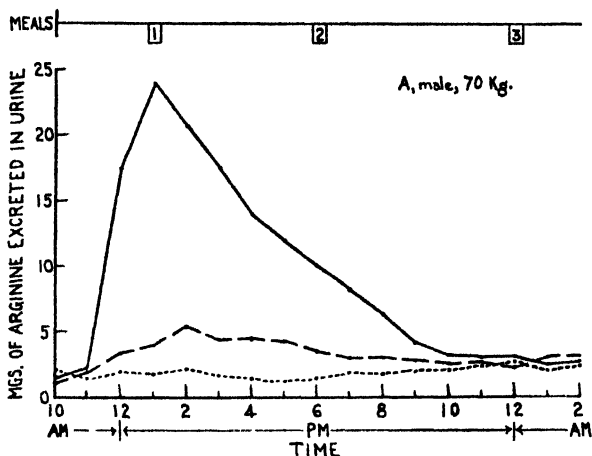


FIG. 1. Urinary output of arginine of Subject A after administration of 0.01 mole (2.1 gm.) of *l*(+)- and *dl*-arginine hydrochloride. The solid line indicates excretion after ingestion of the *dl* form; the broken line after ingestion of the *l* variety; and the dotted line normal output. Meals 1, 2, and 3 had approximately the same composition in all experiments.

of the racemate causes a marked and prolonged elevation of the urinary arginine level. From these experiments it was found that in the 10 to 12 hour period following the ingestion of the racemate 100 to 160 mg. (average 120 mg.) more of arginine are excreted than when *l*(+)-arginine is given. Inasmuch as this excess urinary arginine appears to be excreted only after the feeding of 1.74 gm. of *dl*-arginine which contains 0.87 gm. of *d*(-)-arginine, it is likely to be of the *d* configuration. With the exception of the elevated arginine output, measurements of other nitrogen metabolites following the administration of *dl*-arginine failed to reveal any anomalies which might account for the presence of the *d* isomer. Complete analytical data of one of the five experiments are given in Table I. The lack of a significant difference in the amino N output in these experiments points



to the possibility that the *d*(-)-ornithine which would be derived metabolically from the unexcreted  $740 \pm 30$  mg. of *d*(-)-arginine may also be metabolized by the human.

*In Vitro Experiments*—Arginase-containing extracts of liver were prepared by the procedure of Hunter and Dauphinee (8) from 72 gm. of pooled rat livers (five rats) and from 167 gm. of human liver secured at autopsy from a 51 year-old male Negro 8 hours after death caused by acute pneu-

TABLE I

*Urinary Output of Nitrogen Compounds after Ingestion of 2.1 Gm. of l(+)- and dl-Arginine Hydrochloride by Fasting Normal Adult Human\**  
Subject A, male, 70 kilos.

Form fed	Time after ingestion	Urine volume	Total N	Amino N	Arginine	Urea	Creatine	Creatinine
	hrs.	cc.	mg.	mg.	mg.	mg.	mg.	mg.
<i>l</i> (+)-Arginine	0	345	452	11.7	2.6	894	23.8	63.5
	1	130	534	11.9	3.9	1016	30.3	83.8
	2	180	570	18.3	3.4	954	13.3	52.1
	3	110	555	12.3	2.1	850	11.3	60.1
	4	164	344	11.3	5.6	676	17.7	62.4
	5	60	296	12.8	4.6	636	13.8	63.4
	6	38	400	10.8	4.5	594	16.0	68.5
Total		682	2699	77.4	24.1	4726	102.4	390.3
<i>dl</i> -Arginine	0	450	624	12.9	1.2	1120	20.2	90.0
	1	250	624	14.9	2.9	1330	26.0	88.5
	2	100	457	16.8	16.2	968	14.9	62.7
	3	70	395	11.2	22.4	625	15.7	60.5
	4	55	364	13.6	21.4	654	25.7	67.2
	5	75	281	11.0	19.9	476	11.2	56.0
	6	75	426	12.1	16.8	652	17.9	67.2
Total		625	2547	79.6	99.6	4705	111.4	402.1

\* The total values do not include the zero hour output.

monia. The following assay procedure was employed. 1 cc. aliquots of solutions containing 1.3 to 1.4 mg. of *dl*- or *l*(+)-arginine hydrochloride adjusted to pH 8 and 1 cc. of the arginase preparations also at pH 8 were incubated for 4 to 16 hours at 37°. At the end of this period, 1 cc. samples of the reaction mixtures were transferred to 15 cc. conical centrifuge tubes containing 3 cc. of 10 per cent trichloroacetic acid. After the tubes were shaken and allowed to stand for 10 minutes, they were centrifuged for 5 minutes at 3000 R.P.M. The urea was determined colorimetrically as described by Barker (6) in 1 cc. aliquots of the supernatant fluid. The

urea content of the arginase preparations was similarly estimated in blank determinations and the values were interpolated into the calculations.

The results of these experiments are given in Table II. These data indicate clearly that the extract of human liver, unlike the preparation from rat liver, reacts to produce urea quantitatively from both *d*(-)- and *l*(+)-arginine. This surprising finding was confirmed by repeated experiments and further checked by the negative Sakaguchi tests (4) given by the protein-free filtrates obtained from the reaction mixtures. It is to be further noted that, whereas the reaction with human arginase was quanti-

TABLE II

*Action of Crude Arginase from Human and Rat Livers on l(+)- and dl-Arginine*

Each final value represents the average of ten tests in which 1 cc. of arginine was incubated at pH 8 and 37° with 1 cc. of arginase preparation.

Reactants			Re- action time	Urea formed		
Arginase source and urea blank	Arginine-HCl			Calcu- lated	Found	Yield
	Form	Amount				
		mg.	hrs	mg	mg.	per cent
Human liver, 0.464 mg. of urea per cc.	<i>l</i> (+)-	1.390	4	0.398	0.403 ± 0.005	101.2 ± 1.3
	<i>dl</i> -	1.400	4	0.400	0.408 ± 0.010	101.8 ± 2.0
	<i>l</i> (+)-	1.300	16	0.372	0.375 ± 0.005	99.5 ± 1.2
	<i>dl</i> -	1.400	16	0.400	0.398 ± 0.010	99.4 ± 1.8
Rat livers, 0.260 mg. of urea per cc.	<i>l</i> (+)-	1.400	4	0.400	0.336 ± 0.010	84.0 ± 1.0
	<i>dl</i> -	1.400	4	0.400	0.194 ± 0.008	48.5 ± 2.0
	<i>l</i> (+)-	1.400	16	0.400	0.406 ± 0.002	101.5 ± 0.4
	<i>dl</i> -	1.400	16	0.400	0.194 ± 0.003	48.5 ± 0.6

tatively completed in 4 hours, the reaction with rat arginase required 16 hours for completion.

#### DISCUSSION

The *in vitro* experiments reported here indicate that in human liver, but not in rat and calf (9) liver, there occurs a mechanism whereby urea is produced from both *d*(-)- and *l*(+)-arginine. If one makes the reasonable assumption that this biochemical system also exists in the living man, then the fate of approximately 500 mg. of *d*(-)-ornithine which would result from the transformation of the unexcreted  $740 \pm 30$  mg. of *d*(-)-arginine must be considered. And if the *d*(-)-ornithine were not utilized, then the amino N output following the administration of *dl*-arginine would be expected to be a calculated 53.0 mg. greater than after *l*(+)-arginine ingestion. Inasmuch as our data fail to reveal any significant difference in output of this urinary component, it seems permissible to infer that *d*(-)-ornithine

is available to the human. Furthermore, since ornithine does not appear to be a constituent of proteins and since arginine isolated from proteins always appears to be of the *l*(+) configuration, it must be concluded that, prior to final utilization, *d*(-)-ornithine must be converted to the *l* isomer. Apparently mechanisms for the optical conversion of this and other amino acids exist in man which differ qualitatively and quantitatively from those prevailing in other mammals (10).

These investigations on the human utilization of the *d* forms of the amino acids are being continued and experiments with histidine, tyrosine, and threonine will be reported subsequently.

#### SUMMARY

A comparison of the output of N metabolites, urea, amino N, and arginine in particular, following the administration of equal quantities of *l*(+)- and *dl*-arginine suggests that the *d* form is catabolized in the human. This suggestion receives experimental support from the finding that crude arginase prepared from human liver yields stoichiometric equivalents of urea from both *d*(-)- and *l*(+)-arginine. Experiments with rat liver preparations demonstrated a lack of arginase activity with respect to *d*(-)-arginine.

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# THE UTILIZATION OF *d*-AMINO ACIDS BY MAN\*

## IV. ACETYLTRYPTOPHANE

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In attempting to secure data on the formation in the human of the aberrant metabolite of *d*-tryptophane, it occurred to us that relevant information might be obtained from the feeding of the acetyl derivatives of *l*- and *dl*-tryptophane. As in our previous studies, the subjects were given 0.01 mole quantities of these compounds and their metabolic fate deduced from measurements of urinary products. These investigations unexpectedly revealed that urines collected after administration of acetyl-*dl*-tryptophane, in contrast to those after *dl*-tryptophane, did not yield indigo red on treatment with 0.1 N iodine solution (1). This observation and the measurements of other indole derivatives suggested that a greater amount of acetyl-*dl*-tryptophane than *dl*-tryptophane may be available to man.

### EXPERIMENTAL

Commercially available *l*(-)-tryptophane, Merck (13.5 per cent N found), and *dl*-tryptophane, Merck (13.6 per cent N found), were used in these experiments. The acetyl derivatives of these compounds were prepared in this laboratory by the method of du Vigneaud and Sealock (2). Thus, 14.9 gm. of acetyl-*l*-tryptophane were obtained from 13.6 gm. of *l*-tryptophane which was found to contain 11.30 per cent N by micro-Kjeldahl analysis (3) (theory, 11.38 per cent N) and the specific rotation of a 1 per cent solution in 1 N NaOH was  $[\alpha]_D^{31} = +28.9^\circ$  and  $[\alpha]_D^{20} = +22.0^\circ$  (du Vigneaud and Sealock (2)  $[\alpha]_D^{31} = +29^\circ$ ). The acetylation of 20 gm. of *dl*-tryptophane yielded 20 gm. of the derivative which contained 11.36 per cent N by micro-Kjeldahl analysis and was optically inactive.

In accordance with our adopted experimental technique, fasting adult subjects were given 0.01 mole (2.46 gm.) of acetyl-*l*- or acetyl-*dl*-tryptophane or 0.01 mole (2.04 gm.) of *l*- or *dl*-tryptophane in 240 cc. of water 2 hours after a light breakfast and 120 cc. of water at the end of each

\* The work described in this report was supported in part by grants from the Rockefeller Foundation and the Nutrition Foundation, Inc. Partial support was also derived under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Johns Hopkins University.

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of the subsequent 5 hours to maintain uniform urine flow. Since it had been previously found that food intake did not appreciably affect the output of tryptophane or other indole compounds, the fast was imposed only for the first 3 hours of the 6 hour experiments. The urine was collected at zero time<sup>1</sup> and hourly thereafter and analyzed for tryptophane (4), the *d*-tryptophane metabolite (1), and indican (5).

The experiments revealed that the urine collected after the ingestion of acetyl-*dl*-tryptophane, unlike that after *dl*-tryptophane, failed to yield indigo red on treatment with iodine solution. Urines from experiments with

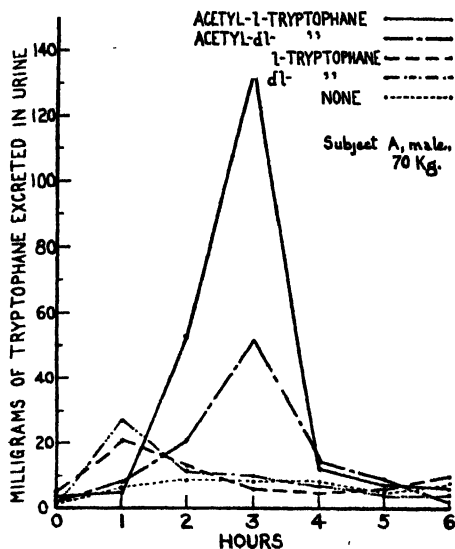


FIG. 1. Urinary output of tryptophane of a normal fasting subject and after administration of 0.01 mole of the isomers of tryptophane and acetyltryptophane.

acetyl-*l*-tryptophane as with *l*-tryptophane also gave negative tests with iodine. The indican output in all instances was found to fall within the normal limits. Tryptophane excretion measurements (Fig. 1), however, showed that a greater loss of the amino acid was incurred by the administration of the acetyl derivatives of the isomers than by tryptophane itself.

Since acetylated tryptophane was previously found to respond negatively to our tryptophane reaction (4) and since some of the ingested acetyltryptophane might well be excreted as such, its presence in the urines was tested by performing the analyses before and after hydrolysis of the specimens under a reflux for 3 hours in the presence of 10 per cent Ba(OH)<sub>2</sub>.

<sup>1</sup> The urine which was collected for the 2 hour period prior to administration of acetyltryptophane is designated as urine collected at zero time.

The failure of this treatment to increase or change the tryptophane content of these samples was taken to indicate the absence of acetyltryptophane.

In performing these analyses it was observed that all of the mercury precipitates obtained from the urines collected after acetyl-*l*- or acetyl-*dl*-tryptophane administration were a bright yellow color instead of the usual gray of normal specimens. The reason for this difference could not be ascertained.

In six experiments an average of  $210 \pm 10$  mg. of tryptophane was excreted following the ingestion of 0.01 mole of acetyl-*l*-tryptophane, whereas only  $95 \pm 5$  mg. of tryptophane were excreted after 0.01 mole of acetyl-*dl*-tryptophane. Since the racemate provides the subject with only one-half the amount of acetyl-*l*-tryptophane as in the acetyl-*l*-tryptophane

TABLE I

*Utilization of Optical Isomers of Tryptophane and Acetyltryptophane by Mouse, Rat, and Man*

Compound	Mouse	Rat	Man
<i>l</i> -Tryptophane	+	+	+
<i>d</i> -Tryptophane	$\pm$ (6, 7)	+	—
Acetyl- <i>l</i> -tryptophane		+	+
Acetyl- <i>d</i> -tryptophane		— (9)	+

The figures in parentheses represent bibliographic reference numbers.

experiments, the order of magnitude of the total tryptophane excretion values suggests that the phenomenon is caused predominantly by acetyl-*l*-tryptophane. This may be due to a low kidney threshold value for this compound.

#### *Comment*

The results reported here indicate that, with the exception of a 5 per cent urinary loss, all of the acetyl-*dl*-tryptophane may be available to man. This is in sharp contrast with the 50 per cent utilization of *dl*-tryptophane previously noted (1). This interpretation of the findings recommends the use of acetyl-*dl*-tryptophane (or better its readily soluble sodium salt) rather than *dl*-tryptophane in the reinforcement of acid hydrolysates of proteins now being offered for supplemental alimentation.

Deductions made from the available data on the metabolism of the isomers of tryptophane and acetyltryptophane in the mouse, rat, and man are compiled in Table I. In making this comparison it is to be pointed out that, in the cases of the mouse and rat, actual growth was employed as the criterion of utilization, whereas in the case of man the biological value of these substances was determined by measurements of urinary products.

The metabolic idiosyncrasies of these mammals which are thus revealed clearly indicate the desirability of studying the availability of the unnatural amino acids and their derivatives directly in man, rather than relying on inferences based on animal experiments.

Finally it appears from this investigation that the metabolic deacetylation of acetyl-*d*-tryptophane results in the formation of an indole derivative which, unlike that formed in the metabolism of *d*-tryptophane, is available to man.

#### SUMMARY

It has been observed that the metabolism of the *d* component of acetyl-*dl*-tryptophane, unlike that of *dl*-tryptophane, does not differ qualitatively from that of the *l* component in the human. Measurements of the urinary output of indole metabolites following the administration of acetyl-*l*(-)- and acetyl-*dl*-tryptophane point to the greater utilization of acetyl-*d*-tryptophane in man than of *d*-tryptophane. The significance of these observations to human nutrition is discussed.

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# MICROBIOLOGICAL METHODS FOR THE DETERMINATION OF AMINO ACIDS

## II. A UNIFORM ASSAY FOR THE TEN ESSENTIAL AMINO ACIDS

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Microbiological methods for the determination of amino acids, because of their specificity, accuracy, sensitivity, and ability to yield many replicate results within a short time, promise to become of increasing importance in investigations of the chemistry and biochemistry of proteins and amino acids. Although such methods are of relatively recent origin, a considerable and rapidly increasing number are already available for the assay of many of the amino acids in purified proteins and natural products. Since a previous review of the literature (1), a method for lysine (2) and additional methods for tryptophane (3) and glutamic acid (4, 5) have been published. Also a fungus method is available for the assay of leucine (6).

With the exception of leucine (6-8), isoleucine (7), valine (7, 8), and tryptophane (3, 9), microbiological methods have either not been developed or existing methods have not been shown to be applicable to the determination of the essential amino acids in meats, grains, milk, and other natural materials. It is in the analysis of such substances rather than purified proteins that microbiological methods will find their most extensive application. Also the analysis of a protein for those amino acids which can be measured by established microbiological methods involves use of a variety of microorganisms, media, and details of procedure. This is confusing and troublesome even to those who have considerable experience with microbiological assay methods. Experience with the latter suggested that a large degree of standardization and, therefore, simplification of amino acid methods was possible and highly desirable. The present paper describes a basic method for the assay of the ten essential amino acids, namely histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, tryptophane, and phenylalanine, which is applicable to foodstuffs and other natural products as well as to purified proteins and synthetic amino acid mixtures. A complete analysis can be made with 1.5 gm. or less of sample. Nine of the amino acids are determined with *Streptococcus faecalis* and phenylalanine with *Lactobacillus delbrückii* LD5.<sup>1</sup> Although two organisms

<sup>1</sup> These organisms can be obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C., where *Streptococcus faecalis* (also known as *Streptococcus lactis* R) is listed as No. 9790 and *Lactobacillus delbrückii* LD5 as No. 9595.



are used, only one standard medium and one procedure are employed. The response of the two organisms to the amino acids is measured by titrating, with standard alkali, the lactic acid produced during growth.

### Procedure

The described procedure applies to both *Streptococcus faecalis* and *Lactobacillus delbrückii* except for two indicated minor details and is similar in most respects to that already outlined (1).

TABLE I  
Basal Medium\*

<i>dl</i> -Leucine	100 mg.	Sodium acetate (anhydrous).	3 gm.
<i>dl</i> -Isoleucine	100 "	Adenine	5 mg.
<i>dl</i> -Valine	100 "	Guanine	5 "
<i>l</i> (-)-Cystine	100 "	Uracil	5 "
<i>dl</i> -Methionine	100 "	Pantothenic acid	100 γ
<i>dl</i> -Tryptophane	200 "	Riboflavin	100 "
<i>l</i> (-)-Tyrosine	100 "	Thiamine HCl	100 "
<i>dl</i> -Phenylalanine	100 "	Nicotinic acid	100 "
<i>dl</i> -Glutamic acid	100 "	Pyridoxamine†	200 "
<i>dl</i> -Threonine	100 "	<i>p</i> -Aminobenzoic acid	20 "
<i>dl</i> -Alanine	100 "	Biotin	0.1 "
<i>dl</i> -Aspartic acid	100 "	Folic acid‡	1.0 "§
<i>l</i> (+)-Lysine	50 "	Salts A	
<i>l</i> (+)-Arginine	100 "	K <sub>2</sub> HPO <sub>4</sub>	250 mg.
<i>l</i> (+)-Histidine	100 "	KH <sub>2</sub> PO <sub>4</sub>	250 "
<i>dl</i> -Serine	100 "	Salts B	
<i>l</i> (-)-Proline	100 "	MgSO <sub>4</sub> ·7H <sub>2</sub> O	100 "
<i>l</i> (-)-Hydroxyproline	100 "	NaCl	5 "
<i>dl</i> -Norleucine	100 "	FeSO <sub>4</sub> ·7H <sub>2</sub> O	5 "
Glycine	100 "	MnSO <sub>4</sub> ·4H <sub>2</sub> O	5 "
Glucose	5 gm.	Adjust to pH 6.8	
		Add distilled H <sub>2</sub> O to.	250 cc.

\* The amino acid being assayed is omitted from the medium.

† Sold by Merck and Company, Inc.

‡ Obtainable from Dr. R. J. Williams, University of Texas, Austin, Texas.

§ Equivalent to 1.0 γ of material of "potency 40,000."

**Inoculum**—The cells are prepared according to previous directions (1) except that *Streptococcus faecalis* is suspended in 100 cc. of water after being centrifuged and washed. Although one experiment indicated that *Lactobacillus delbrückii* also could be diluted to the same degree, most of the assay media were inoculated with cells suspended in 20 cc. of water.

**Assay Medium**—The composition of the basic assay medium is shown in Table I. The amino acid which is being determined is omitted from the

medium. Stock solutions of the amino acids, salts, and vitamins are prepared as indicated previously (1) with the following changes and additions. Owing to difficulty in obtaining *l*(-)-tryptophane, the *dl* form is used. Because of its lesser solubility, stock solutions are prepared with 0.2 N HCl to contain 20 mg. of *dl*-tryptophane per cc. A stock solution containing 1 mg. per cc. of adenine, guanine, and uracil is prepared by dissolving 870 mg. of adenine sulfate, 620 mg. of guanine hydrochloride, and 500 mg. of uracil in 200 cc. of water containing 10 cc. of concentrated HCl and adjusting the volume to 500 cc. Individual aqueous solutions containing per cc. 100  $\gamma$  of thiamine hydrochloride and 10  $\gamma$  of *p*-aminobenzoic acid are also required. A solution of pyridoxamine dihydrochloride is prepared in a concentration of 100  $\gamma$  of pyridoxamine per cc., in place of the more dilute solution previously described, to avoid excessive dilution of the basal medium. It is stable for at least a month when preserved with toluene and stored in the refrigerator.

*Preparation of Samples for Assay*—Fresh, moist substances such as vegetables or meats are sliced and dried at 100° sufficiently to permit grinding into a homogeneous mass. It is not necessary to free the samples of fatty materials or any other constituent prior to hydrolysis or assay. 1 gm. of dried impure protein is autoclaved with 10 cc. of 10 per cent HCl<sup>2</sup> in sealed ampuls of 20 cc. capacity for 10 hours at 15 pounds pressure and prepared for assay as outlined earlier (1). This quantity should be sufficient for determining all of the essential amino acids except tryptophane which requires a separate alkaline hydrolysis. With purified proteins and other materials available only in small quantities, the amount hydrolyzed may be reduced considerably, the degree depending upon the amino acid content of the sample. This can be calculated, approximately, from the sensitivity of each assay as indicated by the standard curves (Fig. 1). Use of smaller samples is possible also if only some of the essential amino acids are to be determined. A further reduction should be possible by decreasing, proportionately, the scale of the assay from 10 cc. total volume per assay tube to 2.5 cc. or less (10, 11).

For the determination of tryptophane, 0.5 gm. of sample is hydrolyzed with 10 cc. of 5 N NaOH in 50 cc. Pyrex Erlenmeyer flasks plugged with non-absorbent cotton. Hydrolysis in sealed ampuls should also be satisfactory and perhaps preferable, since appreciable amounts of water sometimes enter the flasks during autoclaving. In the case of purified proteins, 25 mg. are hydrolyzed with 2 cc. of alkali in sealed ampuls. The quantity of sample may be reduced as indicated above for acid hydrolysis. Digestion is carried out at 15 pounds steam pressure for 10 hours as for acid hy-

<sup>2</sup> Concentrated HCl (about 38 per cent) is diluted with water to 10 per cent, by volume, of HCl.

drolysis so that both types of digestion may be made simultaneously in one autoclave. Frequently a copious precipitate appears on neutralization of the alkaline digest which may contain considerable quantities of silica dissolved from the flask. The precipitate is best removed by centrifuga-

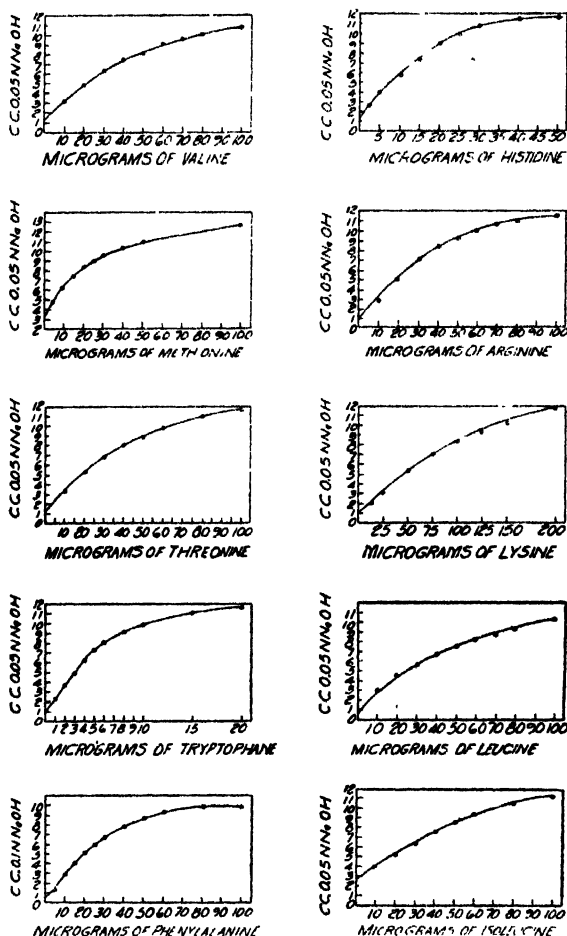


FIG. 1. Typical standard curves. The quantities of each amino acid are those of the *l* or naturally occurring isomer.

tion, since filtration through paper may be extremely slow. It is washed once with water which is combined then with the remainder of the precipitate-free hydrolysate.

*Activity of Optical Isomers and Preparation of Standards*—With the exception of histidine of which only the *l* isomer was available, the synthetic

*dl* racemate of each amino acid, under our conditions, was exactly one-half as active as the *l* isomer, indicating that the *d* or unnatural isomer is inactive (12). Data for threonine, which is typical of all the amino acids, are given in Table II. Identical standard curves were obtained with the *l* and *dl* forms when twice as much of the latter as compared to the *l* isomer was used. Therefore, either form can be used as the standard. It is important, obviously, that amino acids of known purity be employed. It may be necessary to purify commercial *dl*-leucine (13) and *dl*-isoleucine.

It is convenient to prepare solutions of the amino acids, to be used as standards, in a concentration of 100  $\gamma$  per cc. of the *l* isomer. These may be stored under toluene in the refrigerator. Dilutions are made from these primary solutions so that the quantities needed for construction of the standard curves (Fig. 1), which are in terms of the *l* isomer, can be pipetted

TABLE II  
Activity of Threonine Enantiomorphs for *Streptococcus faecalis*

Isomer per tube	<i>l</i> (-)	<i>dl</i> -	<i>d</i> (+)-	<i>dl</i> -Allo-
	0.05 N acid formed per tube			
mg.	cc.	cc.	cc.	cc.
0	0.9, 0.9			
0.01	3.6, 3.3			
0.02	5.1, 5.2	3.7, 3.4		
0.04	7.9, 7.9	5.2, 5.2		
0.08		8.0, 8.0		
2.0			0.9, 0.9	1.5, 1.5

conveniently. These secondary dilutions, also, may be preserved. The dilutions must be made so that the maximum volume of standard added to the assay tubes does not exceed 5 cc.

**Assay Procedure**—This is identical with that already outlined (1) except for the following minor changes applicable only to assays with *Streptococcus faecalis*; there are no changes for the assay of phenylalanine with *Lactobacillus delbrückii*. *Streptococcus faecalis* assays are titrated with approximately 0.05 N NaOH after 40 hours incubation at which time maximum acid production has occurred. Some experiments have indicated that it may be possible to reduce the incubation period to 16 hours for most routine work and that as an alternative to titration the turbidity of the cultures may be measured with a photoelectric cell. However, the latter can be used only if the sample does not impart any appreciable amount of color or turbidity to the assay medium.

**Recording and Calculation of Results**—An example of a convenient method is the assay of beef liver for threonine. 1 gm. of dried liver was digested

with 10 cc. of HCl and the hydrolysate neutralized, filtered, and adjusted to 100 cc.; a further 1:12.5 dilution was made prior to assay (Table III).

Alkaline hydrolysis used to liberate tryptophane from proteins results in complete racemization of this amino acid. Since the *d* form is inactive for *Streptococcus faecalis*, assay values must be multiplied by a factor of 2 to arrive at the final correct tryptophane content of the protein.

TABLE III  
*Assay of Beef Liver for Threonine*

Diluted sample added to assay tube	NaOH required to neutralize assay tube after incubation	Amino acid equivalent of titration figure as read from standard curve	Amino acid content calculated for 1 cc. diluted sample at each assay level
cc	cc	γ	γ
0.5	3.6	10	20
1.0	5.1	19	19
1.5	6.8	31	21
2.0	7.85	40	20
3.0	9.6	62	21
Average.			20.2

$$20.2 \times \frac{\text{dilution}}{12.5} \times 100 = 25.25 \text{ mg. of threonine per gm., or 2.53 per cent.}$$

#### DISCUSSION

*Streptococcus faecalis* was selected for use in the assays because, in contrast to the commonly employed *Lactobacillus casei* and *Lactobacillus arabinosus*, its amino acid requirements are not influenced by pyridoxamine or pyridoxal; those members of the vitamin B<sub>6</sub> group can substitute for lysine and threonine in the nutrition of the lactobacilli (14). The latter are unsuitable, therefore, for the assay of lysine and threonine, since pyridoxamine and pyridoxal or closely related compounds are commonly present in natural products (15) or are formed readily in the assay medium during sterilization (14, 16).

Although initial, short time experiments appeared to indicate that *Streptococcus faecalis* required all ten essential amino acids for full growth, subsequent experience disclosed that the almost imperceptible development evident in 16 to 20 hours, in the absence of phenylalanine, rapidly increased to maximum growth and acid production in 40 hours. Inability to obtain cultures from thirty-five single colonies which had an absolute requirement for phenylalanine suggests that *Streptococcus faecalis* is able to synthesize phenylalanine, although too slowly to permit a normal rate of growth. This situation is analogous to that of the rat with respect to arginine (17).

*Lactobacillus arabinosus* also developed without phenylalanine. This was not unexpected, since its need for phenylalanine is dependent, critically, on the composition of the growth medium (18). However, *Lactobacillus delbrückii* LD5, *Lactobacillus casei*, and *Leuconostoc mesenteroides* P-60 failed to grow unless phenylalanine was in the medium and they responded in approximately linear fashion to increments of the amino acid. The former was chosen for development of the phenylalanine assay, because previous experience with it in amino acid assays was available (1).

The basal medium (Table I) resembles, closely, those proposed for other microbial amino acid assay methods. Although the number of ingredients is formidable, most of them are essential for growth. The amino acids and growth factors are in excess both in number and quantity. Thus proline, hydroxyproline, norleucine, and glycine are not essential for the growth of either assay organism and the same applies to thiamine and *p*-aminobenzoic acid and probably also to guanine and uracil. However, such a medium seemed preferable to a "minimum" medium which might be unduly sensitive to non-specific stimulatory substances or inhibitory substances that might be introduced with the sample. Doubling of the glucose and sodium acetate increases acid formation only slightly and has no effect on the assay values.

In the development of the method for lysine, standard curves were obtained frequently which "dipped" in the center in contrast to normal linear curves. The medium in use at the time was one which had been and still was entirely satisfactory for histidine and threonine assays developed earlier. Amino acid values from such irregular curves did not agree closely when calculated from the different assay levels and were high compared to those from assays with normal standard curves. The dip was eliminated by doubling the concentration of adenine, guanine, uracil, and vitamin supplements, except folic acid. Increase of the purine and pyrimidine bases was the most important factor in eliminating the dip, followed by biotin and nicotinic acid. However, the higher concentration of all three was necessary for linear curves. The modified basal medium proved to be satisfactory for the assay of histidine and threonine and also for the remainder of the amino acid assay methods developed subsequently.

The proposed assay method fulfils, for each of the amino acids, the conventional but adequate criteria of reliability: (a) Essentially the same amino acid values are obtained for a particular protein irrespective of the amount of sample assayed, thus indicating that the method is stable to non-specific stimulatory or inhibitory substances which may be introduced with samples (Table IV). This holds for such complex materials as tankage, blood meal, and yeast and also for carrots and potatoes which contain only small amounts of amino acids and must be added, therefore, in relatively large

amounts to the assay medium. (b) The results are readily reproducible, so that the same values are obtained for a given protein in repeated assays with fresh hydrolysates, different batches of medium, and different opera-

TABLE IV

*Amino Acid Content of Proteins at Different Assay Levels*

The results are calculated for partially dried material.

Liver per assay tube	Tryptophane		Tankage per assay tube	Threonine		Peas per assay tube	Valine		Alfalfa meal per assay tube	Methionine	
	Found	Content		Found	Content		Found	Content		Found	Content
mg.	γ	per cent	mg.	γ	per cent	mg.	γ	per cent	mg.	γ	per cent
0.2	1.8	0.90	0.5	9	1.8	0.5	6	1.2	5	1.3	0.026
0.4	3.4	0.85	1.0	18	1.8	1.0	10	1.0	10	2.2	0.022
0.6	5.4	0.90	1.5	28	1.9	2.0	21	1.1	15	3.8	0.025
0.8	7.4	0.95	2.0	40	2.0	3.0	35	1.2	20	5.0	0.025
1.2	10.4	0.85	3.0	59	2.0	4.0	47	1.2	30	7.5	0.025
Average		0.89			1.90			1.14			0.025

TABLE V

*Reproducibility of Amino Acid Values*

The results are calculated in per cent, on a dry basis.

Amino acid	Protein	Assay 1	Assay 2	Assay 3	Assay 4	Mean
Lysine	Casein	7.7	7.8	7.6		7.7
	Soy bean flour, defatted	3.2	3.2	3.4	3.5	3.3
	Blood meal	8.2	8.5	8.4	8.4	8.4
	Tankage	4.7	4.9	4.8	4.8	4.8
Isoleucine	Yeast, brewers'	2.4	2.4	2.4		2.4
	Tankage	1.75	1.74	1.89		1.79
	Silk fibroin	1.07	1.17	1.13		1.12
Methionine	Wheat, seed	0.15	0.17	0.17		0.16
	Blood meal	1.01	1.01	1.04		1.02
	Tankage	0.84	0.87	0.86		0.86
	Gelatin	0.59	0.58	0.59		0.59
Threonine	Liver, beef	3.1	3.1	3.2		3.1
	Peas	1.17	1.17	1.24		1.19
	Rye, seed	0.36	0.36	0.38		0.37
	Blood meal	3.8	3.8	3.8		3.8
	Tankage	2.1	2.0	2.0		2.0

tors (Table V). (c) Recoveries of known amounts of amino acids added to proteins prior to hydrolysis are quantitative, generally within  $\pm 2$  per cent (Table VI). (d) Compounds related chemically or physiologically to the amino acids, other than a few very closely related exceptions, are inactive

(Table VII). Thus ornithine and citrulline which can individually replace arginine in the nutrition of certain *Neurospora* mutants (19) and the chick (20) are unable to do so for *Streptococcus faecalis*. Likewise, the combination of choline plus homocystine which can substitute for methionine in the growth of the rat (21) and the chick (22) is inactive for *S. faecalis*. Also, indole and anthranilic acid which can replace tryptophane for *Lactobacillus arabinosus* (9, 23) and *L. casei* (23) are inactive for *S. faecalis*. This last observation simplifies the present tryptophane method, since samples do not need to be extracted with ether and toluene to remove indole and anthranilic

TABLE VI

*Recovery of Amino Acids Added to Proteins Prior to Hydrolysis*

The results are calculated in mg. per gm. of partially dried material.

Amino acid	Substance	Con- tent	Added	Total	Found	Per cent recov- ery
Arginine	Blood meal	36	39.4	75.4	76	101
	Soy bean flour, defatted	38	39.4	77.4	72	93
	Gelatin	80	83	163	162	99
Valine	Casein	65.6	61.5	127.1	124	98
	Blood meal	68.1	65	133.1	130	98
	Yeast, brewers'	27.5	30	57.5	59.7	104
Leucine	Soy bean flour, defatted	40.4	45	85.4	84.2	99
	Blood meal	104	100	204	204	100
	Gelatin	31.3	35	66.3	65.3	99
Phenylalanine	Casein	56.5	50	106.5	107	100
	Blood meal	62.5	50	112.5	113	100
	Yeast, brewers'	23.5	25	48.5	50.4	104
Threonine	Casein	42.8	40	82.8	80.8	98
	Gelatin	18.8	20	38.8	39.5	102
	Blood meal	35.8	40	75.8	74.3	98
	Yeast, brewers'	29.0	30	59.0	57.1	97
	Soy bean flour, defatted	20.8	20	40.8	40.4	99

acid as is the case with one of the *L. arabinosus* methods (9). (e) In general, the microbiological values for purified and impure proteins are in reasonably good agreement with those obtained by the more recent, improved chemical methods. The degree of agreement for lysine is characteristic of all of the essential amino acids with the exception of methionine (Table VIII) for which lower microbiological values were obtained, consistently. In view of the remarkable specificity of the microbial response to methionine (Table VII) and also to the other amino acids, it seems possible that the higher chemical values may include substances other than methionine. For further comparisons of the microbiological values for the purified proteins and



natural products listed in Tables IX and X, with chemical data, reference can be made to the recent extensive compilations by Block and Bolling (36). If desired, the amino acid values in Table X can be recalculated to express

TABLE VII

*Activity of Compounds Related Chemically or Physiologically to the Essential Amino Acids*

Assay	Inactive*	Active	Per cent activity†
Histidine	Histamine, imidazole		
Arginine..	Ornithine, creatine, creatinine, <i>dl</i> -citrulline	Arginine flavinate	36.5
Lysine	Benzoyl- <i>dl</i> -lysine, $\epsilon$ -benzoylamino-caproic acid, $\epsilon$ -benzoylamino- $\alpha$ -bromocaproic acid		
Leucine.....		<i>l</i> (-)-Leucine, 3-naphthalene sulfonate	35.5
		$\alpha$ -Bromoisocaproic acid	19.5
Isoleucine.	No compounds tested		
Valine		Formyl <i>l</i> (+) valine	2.5
Methionine..	Creatine, creatinine, choline, carboxymethylcysteine, choline + carboxymethylcysteine, <i>dl</i> -homocystine, choline + <i>dl</i> -homocystine, <i>l</i> -cystathionine, choline + <i>l</i> -cystathionine, taurine		
Threonine	<i>o</i> -Methyl- <i>dl</i> -threonine, formyl- <i>o</i> -methyl- <i>dl</i> -threonine, N-benzoyl- <i>dl</i> -threonine, N-benzoyl- <i>o</i> -methyl- <i>dl</i> -threonine, $\alpha$ -bromo- $\beta$ -methoxybutyric acid		
Tryptophane	Benzoyl- <i>dl</i> -tryptophane, indolealdehyde, 2-carboxyindole, 2-carbethoxyindole, indole, anthranilic acid	<i>dl</i> -Tryptophane methyl ester	50
Phenylalanine		$\alpha$ -Bromo- $\beta$ -phenylpropionic acid	67

\* Less than 0.25 per cent (weight basis).

† Weight basis.

percentages of dry weight of sample by multiplying each value by the factor (per cent nitrogen)/16.

Investigation of the effect of 5 to 30 hours of acid hydrolysis on liberation of each of the essential amino acids, except tryptophane, from representative protein materials indicates that autoclaving for 5 hours is sufficient to give

TABLE VIII

*Comparison of Microbiological Amino Acid Values of Proteins with Those Cited in Literature*

Protein		Lysine			Methionine		
		Per cent of dry weight	Per cent of dry weight, literature values	Bibliographic reference	Per cent of dry weight	Per cent of dry weight, literature values	Bibliographic reference
Purified	Casein	7.7	6.25, 6.5, 8.3	(25, 26, 2)	2.6	3.17, 2.86	(27, 28)
	Gelatin	5.8	5.9	(29)	0.59	0.8,* 1.13	(30, 31)
	Egg albumin	6.6	5.06	(32)	4.1	5.25, 4.58	(27, 28)
	$\beta$ -Lactoglobulin	11.1	10.6, 9.9	(33, 34)	2.5	3.22	(24)
	Silk fibroin	0.72	0.25, 0.6	(35, 2)	0.15	0	(36)
	Tobacco mosaic virus	1.36	0.0, 1.35	(37, 38)	<0.06	0.0	(37)
Impure	Rye, seed	4.2*	4.5, 5.2	(36)	1.26*	2.3, 2.7	(36)
	Flour, patent	2.2*	1.9	(36)	0.96*	3	(36)
	Soy bean flour, defatted	5.4*	5.4	(30)	0.84*	2.0	(30)
	Linseed meal	3.3*	2.5	(36)	0.81*	3	(36)
	Alfalfa "	4.9*	4.2	(36)			
	Yeast	6.4*	6.4	(30)	1.37*	2	(36)
	Whole milk	8.7*	7.5	(30)	2.1*	2.8	(30)
	Blood meal	8.8*	6.2, 6.7, 7.7	(36)			
	Tankage	7.2*	6.0	(30)	1.28*	3	(30)
	Liver, beef	6.1*	6.02	(39)	2.0*	2.4, 2.9	(39)

\* Calculated to 16 per cent nitrogen.

TABLE IX

*Amino Acid Content of Purified Proteins*

The results are in per cent, calculated for oven-dried (105°) material.

Protein	Histidine	Arginine	Lysine	Leucine	Isoleucine	Valine	Methionine	Threonine	Tryptophane	Phenylalanine
Casein, S. M. A. . . . .	2.8	3.9	7.7	9.9	5.6	6.7	2.6	4.2	1.07	5.9
Gelatin, Knox . . . . .	0.58	9.1	5.8	3.5	1.72	2.7	0.59	2.0	0.021	2.3
Egg albumin . . . . .	2.3	5.9	6.6	9.2	7.0	7.0	4.1	3.6	1.41	7.9
$\beta$ -Lactoglobulin . . . . .	1.50	2.8	11.1	15.3	7.0	5.5	2.5	4.6	2.1	4.3
Silk fibroin . . . . .	0.41	1.11	0.72	0.93	1.15	3.5	0.15	1.36	0.44	1.49
Tobacco mosaic virus . . . . .	<0.02	8.9	1.36	7.5	5.7	7.0	<0.06	8.7	2.3	6.8

maximum amino acid values. The data in Table XI are typical. For some of the proteins, there is evidence of slight destruction or racemization of some of the amino acids after 30 hours. The results are in accord with

similar microbiological data of other investigators (7, 8, 10) and emphasize, unequivocally, in contrast to conflicting chemical data (36), the marked stability of the amino acids to acid hydrolysis. Since periods of hydrolysis considerably in excess of those required for maximum liberation of amino acids from proteins cause no apparent destruction, the data support the view that the amino acid values of protein hydrolysates are the same as those of the intact proteins.

TABLE X  
*Amino Acid Content of Natural Products*

Substance	Nitrogen	Per cent, calculated to 16 per cent nitrogen, on dry basis									
		Histidine	Arginine	Lysine	Leucine	Isoleucine	Valine	Methionine	Threonine	Tryptophane	Phenylalanine
	<i>per cent of dry weight</i>										
Rye, seed	1.95	1.72	4.3	4.2	6.2	4.0	5.0	1.26	3.0	1.31	5.6
Wheat, seed	2.22	2.0	4.2	2.9	6.8	3.6	4.5	1.20	2.5	1.37	5.1
Flour, patent	2.28	1.54	3.1	2.2	7.5	3.7	4.2	0.96	2.5	0.98	5.6
Soy bean flour, defatted	9.32	2.3	7.1	5.4	7.4	4.5	4.6	0.84	3.9	1.20	5.3
Linseed meal	6.94	1.50	8.4	3.3	5.3	4.2	5.1	0.81	3.0	1.46	5.2
Alfalfa	2.90	1.21	3.1	4.9	6.6	3.6	4.4	0.15	3.3	1.44	4.1
Carrots	1.30	0.74	0.68	1.14	4.8	2.9	3.4	0.56	2.7	0.24	2.8
Peas	4.76	1.21	8.9	5.0	6.4	4.1	4.0	0.43	3.9	0.71	4.8
Yeast, brewers'	9.14	2.1	4.5	6.4	7.1	4.2	5.4	1.37	5.1	1.05	4.4
Whole milk	4.34	2.4	3.6	8.7	9.9	5.2	6.6	2.1	4.0	1.32	5.3
Blood meal	14.96	5.63	4.2	8.8	12.2	1.13	7.7	1.11	4.1	1.28	7.3
Tankage	10.75	2.4	5.9	7.2	7.7	2.7	5.4	1.28	3.0	0.83	4.2
Liver, beef	12.98	1.87	3.4	6.1	8.3	4.0	5.7	2.0	3.8	1.38	5.3
Per cent of dry weight											
Potatoes, peeled*		0.10	0.37	0.33	0.56	0.29	0.46	0.09	0.37	0.13	0.43

\* The results are not calculated to 16 per cent nitrogen because of difficulty in obtaining concordant nitrogen values.

For tryptophane assays with *Lactobacillus arabinosus*, Ba(OH)<sub>2</sub> (9) or enzymatic digestion of proteins (3) has been recommended. Although irregular results have been reported with NaOH digestion (3), it has proved satisfactory under our conditions. To insure complete liberation and racemization of tryptophane, it is necessary to hydrolyze proteins for at least 10 hours (Table XI) and to limit the sample to an amount which does not contain more than 10 to 15 mg. of tryptophane. There is little danger of exceeding that quantity if not more than 0.5 gm. of impure or 100 mg. of purified protein is used.

The described method is eminently suitable for obtaining many analyses within a short time. One experienced operator can assay six proteins for all essential amino acids in little more than a week. The techniques required are relatively simple although elementary knowledge of bacteriological technique is essential in maintaining purity of stock cultures and preventing contamination of assays.

TABLE XI

*Effect of Time of Hydrolysis on Liberation of Amino Acids from Proteins*  
The results are calculated in per cent, on a dry basis.

Amino acid	Protein	Hrs of hydrolysis*				
		5	10	15	20	30
Threonine	Casein	4.1	4.1	4.1	3.9	3.8
	Blood meal	3.7	3.8	3.7	3.5	3.6
	Yeast, brewers'	3.1	3.0	3.0	2.9	2.7
Histidine	Gelatin	0.59	0.60	0.64	0.58	
	Wheat, seed	0.39	0.36	0.38	0.36	0.38
	Blood meal	5.4	5.5	5.3		5.4
Methionine	Casein	2.6	2.5	2.4	2.3	2.2
	Blood meal	1.00	1.01	1.02	0.97	0.95
	Yeast, brewers'	0.79	0.76	0.77	0.76	0.74
Tryptophane	Casein	1.18	1.16	1.11	1.08	1.04
	Wheat, seed	0.19	0.19	0.17	0.18	0.15
	Liver, beef	1.31	1.08	1.10	1.15	1.02
	Egg albumin		1.41		1.41	
	$\beta$ -Lactoglobulin		2.1		2.1	
	Tobacco mosaic virus		2.4		2.2	2.2
Phenylalanine	Casein	5.7	5.7	5.4	5.5	5.1
	Blood meal	6.9	6.7	6.7	6.6	6.2
	Yeast, brewers'	2.6	2.5	2.5	2.5	2.3

\* Autoclaved at 15 pounds steam pressure (121°).

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and Dr. E. E. Howe, Dr. M. Tishler, and Dr. S. A. Harris, Merck Laboratories, for many of the compounds listed in Table VII.

#### SUMMARY

An accurate, specific, and sensitive microbiological method is described for the determination of the ten essential amino acids, namely histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, tryptophane, and phenylalanine, in foodstuffs and other natural products as well as in purified proteins and synthetic amino acid mixtures. A complete amino acid analysis can be made with 1.5 gm. or less of sample. With only one medium and procedure, nine of the amino acids are determined with *Streptococcus faecalis* and phenylalanine with *Lactobacillus delbrückii* LD5. The response of the two organisms to the amino acids is measured by titrating, with standard alkali, the lactic acid formed during growth. The method yields many replicate results within a short time and lends itself readily to routine use. The quantities of essential amino acids in casein, gelatin, egg albumin,  $\beta$ -lactoglobulin, silk fibroin, tobacco mosaic virus, rye, wheat, patent flour, soy bean flour, whole milk, peas, carrots, potatoes, beef liver, brewers' yeast, blood meal, tankage, alfalfa meal, and linseed meal are presented.

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# A SPECIFIC MICROMETHOD FOR THE COLORIMETRIC DETERMINATION OF GLYCINE IN BLOOD AND URINE\*

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Recently we described a method for the determination of alanine based upon its conversion into acetaldehyde by the action of ninhydrin (1). A method for the measurement of glycine has been devised which involves its conversion by ninhydrin to formaldehyde; the latter is then measured by its reaction with chromotropic acid.

Earlier methods for the measurement of glycine are less sensitive and specific than the one here proposed. In 1902 Fischer (2) described a procedure for the gravimetric determination of glycine by isolating it from protein hydrolysates. Other gravimetric methods were devised by Town (3) and Bergmann and Fox (4). The former added nitranilic acid to a protein hydrolysate containing glycine and weighed the insoluble glyco-coll nitranilate thus obtained. The hydrolysate had to be freed of many inorganic salts which interfered with the precipitation. Bergmann and Fox (4) used trioxalatochromiate to produce an insoluble compound with glycine. All of these methods are too insensitive to be applicable to biologic materials in which the amounts of glycine are very small.

Zimmermann (5) reported a colorimetric method for glycine based upon its reaction with *o*-phthalic dialdehyde. The method was subsequently improved by Klein and Linser (6) and Patton (7). Many substances other than glycine, such as histidine, histamine, arginine, cysteine, tryptophane, and ammonia, gave similar reactions and consequently had to be removed before the addition of the dialdehyde. The sensitivity of the method was limited to a minimum of about 1 mg. of glycine.

The reaction between  $\alpha$ -amino acids and ninhydrin has been clearly

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Shortly before this communication was submitted, a paper by Douglas A. MacFadyen appeared in the March issue of the *Journal of Biological Chemistry*, p. 107, on the measurement of formaldehyde by the use of chromotropic acid. MacFadyen defined conditions under which, by action of ninhydrin and chromotropic acid, 0.96 mole of formaldehyde per mole of glycine could be measured.



elucidated (8, 9). Amino acids are deaminated and converted into aldehydes containing 1 less carbon atom. The basis of the method here described for the determination of glycine is its conversion by ninhydrin into formaldehyde which is then measured by allowing it to react with 1,8-dihydroxynaphthalene-3,6-disulfonic acid (chromotropic acid). This reaction, first described by Eegriue (10), is used by Boyd and Logan (11) in their method for the measurement of serine.

### *Method*

#### *Reagents—*

Ninhydrin solution, 1 per cent (triketohydrindene hydrate, Eastman).

Phosphate buffer, pH 5.5 (glass electrode). 3.5 gm. of tripotassium phosphate are added to 100 ml. of a 20 per cent solution of monopotassium phosphate.

Sulfuric acid, 0.66 N.

Sodium tungstate solution, 10 per cent.

Concentrated sulfuric acid, sp. gr. 1.84.

Chromotropic acid solution, 5 per cent 1,8-dihydroxynaphthalene-3,6-disulfonic acid, Eastman. The solution is stored in a refrigerator in an all-glass bottle and is stable for at least 2 weeks.

*Determination of Glycine in Blood*—5.0 ml. of a protein-free filtrate of blood, prepared in the usual way (1) with sulfuric acid and sodium tungstate solution, are placed in the flask of a Stotz all-glass still (12) containing 2 ml. of phosphate buffer, 1 ml. of ninhydrin solution, and a glass bead. The flask is attached to the condenser, and the contents are distilled rapidly into a test-tube calibrated to 10.0 ml. After about 7 ml. of distillate have been collected, the flask is cooled to room temperature by immersing it in a water bath, and then disengaged. 2 ml. of distilled water are added, and distillation is continued to dryness. The neck of the still is heated gently with the flame to distil the last few drops which may have condensed there. The total distillation time should not exceed 15 minutes. The receiving test-tube is removed, and enough distilled water is added to make a total volume of 10.0 ml. After the contents are thoroughly mixed, 5.0 ml. are pipetted into an Evelyn (13) tube,<sup>1</sup> and while it is being cooled and agitated in an ice bath, 4.0 ml. of concentrated sulfuric acid are added slowly. When the reaction mixture has returned approximately to room temperature, 3 drops (0.1 ml.) of the chromotropic acid solution are added. The test-tube is shaken, corked lightly, and placed in a boiling water bath for 30 minutes. After the solution is cooled, the intensity of color is measured in an Evelyn or Klett photoelectric colorimeter, with a No. 565 or No. 540 filter.

<sup>1</sup> If an Evelyn photometer is not used, a Pyrex test-tube of similar dimensions (15 × 2 cm.) may be substituted.

*Determination of Glycine in Urine*—5.0 ml. of a 1:50 dilution of urine are handled in the same way as blood filtrate. It is necessary to run a blank by repeating the procedure on another 5.0 ml. aliquot of the diluted urine with the substitution of 1 ml. of distilled water for the ninhydrin solution.

#### DISCUSSION

*Absorption Curve<sup>2</sup> of Colored Compound Produced by Interaction of Chromotropic Acid and Formaldehyde Obtained from Glycine (Fig. 1)*—The colored compound which results from the reaction between formaldehyde

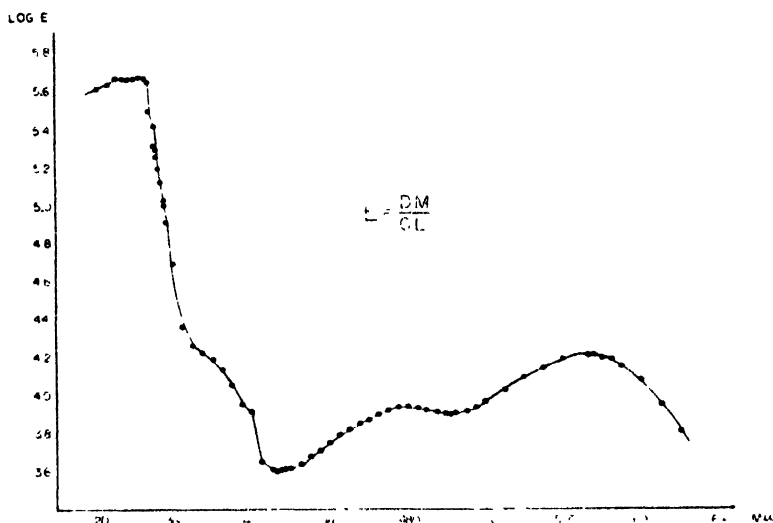


FIG. 1. Visible absorption spectrum of the compound resulting from interaction of chromotropic acid and formaldehyde produced from glycine.  $E$  is the molecular extinction coefficient;  $D$ , optical density;  $M$ , molecular weight (glycine);  $C$ , concentration of glycine (gm. per liter);  $L$ , cell length (cm.)

and chromotropic acid absorbs maximally light of wave-lengths 330, 342, 478, and 575 mμ. The logarithms of the molecular extinction coefficient at these values are 5.678, 5.686, 3.946, and 4.201 respectively.

As might be expected from the above, a No. 565 filter was found to give maximum sensitivity. Since most photoelectric colorimeters are furnished with No. 540 filters, calibration curves with both filters are presented for the Evelyn and Klett colorimeters (Figs. 2 and 3).

#### *Relationship between Color Intensity and Concentration of Formaldehyde*

<sup>2</sup> We are indebted to Dr. William G. Dauben of Harvard University for his assistance in obtaining the absorption curve. A Beckman D. U. photoelectric spectrophotometer was used.

and Glycine (Figs. 2 and 3)—The intensity of color obtained by allowing formaldehyde<sup>3</sup> to react with chromotropic acid was directly proportional to the concentration of the former. This was also true for a pure solution of glycine when subjected to the procedure outlined above. The glycine curve could be superimposed on that obtained for formaldehyde when due allowance was made for stoichiometric difference.

*Sensitivity of Method*—As little as 0.2  $\gamma$  of formaldehyde, representing 0.5  $\gamma$  of glycine, can be measured with the Evelyn photometer. This

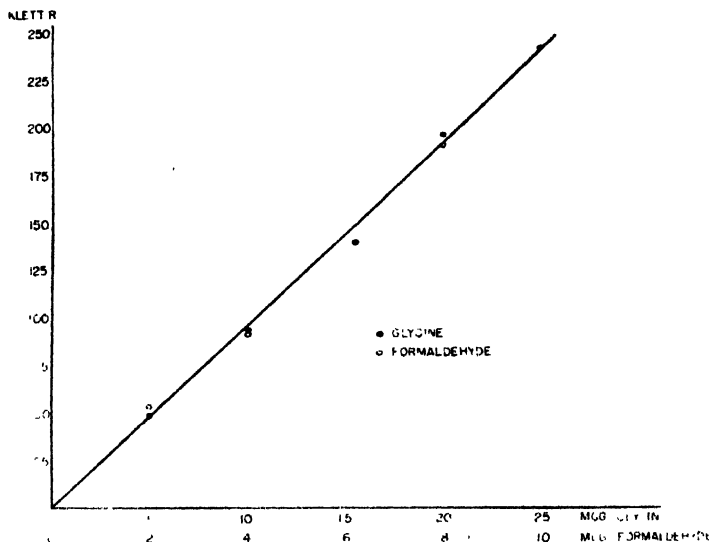


FIG. 2. Relationship between intensity of color and equivalent amounts of formaldehyde and glycine. 540  $m\mu$  filter.

corresponds to a minimum concentration of glycine in blood of 0.2 mg. per cent.

*Specificity of Method*—Egriwe (10) described the remarkable specificity of the reaction between formaldehyde and chromotropic acid. Among many other naturally occurring substances only a few, such as glyceraldehyde, arabinose, fructose, and cane sugar, give any color. The color obtained from them was yellow, whereas the color from formaldehyde is rose-pink. Furfural in high concentrations also gives a pink color. These substances cannot interfere with the determination of glycine as outlined above, because they are not volatile with steam (with the possible exception of furfural).

<sup>3</sup> 200 mg. of trioxymethylene were dissolved in 1 liter of distilled water. Under the influence of the strong sulfuric acid used in the reaction, trioxymethylene was converted quantitatively to formaldehyde.

Boyd and Logan (11) report that 10 per cent of glucosamine can be hydrolyzed by strong mineral acid to give formaldehyde. Although glucosamine may exist in blood, there is no evidence of its breakdown to yield formaldehyde under the conditions of our method. The distillation of urine alone, however, yields a small amount of formaldehyde, as determined with chromotropic acid. Since glucosamine must be hydrolyzed with 7 N hydrochloric acid for 24 hours before as much as 10 per cent of it is broken down to give formaldehyde, it is unlikely that a 15 minute distil-

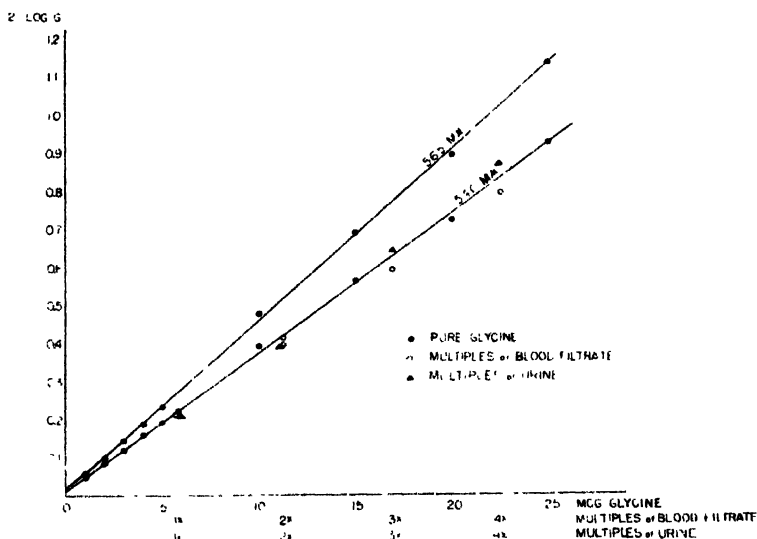


FIG. 3. Calibration curve for glycine obtained with 565 and 540  $m\mu$  filters with the Evelyn photometer. Correlation between analyses of multiples of blood filtrate, of urine, and of glycine.

lation of diluted urine at a pH of 5.5 can result in appreciable amounts of formaldehyde on this basis.

No formaldehyde is obtained when the following substances are subjected to the method for glycine determination: aspartic acid, alanine, tyrosine, lysine, leucine, isoleucine, norleucine, tryptophane, ornithine, threonine, phenylalanine, proline, hydroxyproline, methionine, cystine, cysteine, homocysteine, valine, norvaline, diiodotyrosine, arginine, serine,  $\beta$ -alanine, glutamic acid,  $\alpha$ -aminoisobutyric acid,  $\beta$ -amino-*n*-butyric acid, benzoylalanine.

Alanylglycine interferes to the extent that 5 equivalent parts are required to give the same color intensity as 1 part of glycine. It is unlikely that our alanylglycine was hydrolyzed as a consequence of the reaction, since

TABLE I  
*Duplicability of Glycine Determinations on Blood and Urine*

Material	Specimen No.	Paired glycine determinations		Deviation
		<i>γ per ml.</i>	<i>γ per ml.</i>	<i>per cent</i>
Blood	1	23.8	21.4	10
		44.8*	47.2*	6
		67.6*	67.1*	1
	2	20.0	19.4	3
		65.2*	64.4*	2
	3	20.0	19.5	3
		69.2*	68.0*	2
	4	27.2	27.2	0
		44.0*	44.8*	2
		62.0*	61.6*	1
	5	74.8*	73.2*	3
		41.3*	37.3*	10
59.3*		54.1*	9	
Urine	6	232	232	0
		328*	314*	4
	7	177	171	3
		344*	364*	6
		560*	550*	2
Average . . . . .				4

\* Glycine added.

TABLE II  
*Duplicability of Glycine Determinations on Aliquots of Same Blood*

Glycine	Deviation from mean
$\gamma$ per ml.	per cent
26.4	+4
24.2	-4
26.2	+4
25.4	0
23.2	-8
25.4	0
25.2	0
24.5	-3
27.0	+7
25.3 (Mean)	$\pm 3$ (Average)

we were unable to detect any alanine in the reaction mixture. Accordingly we suspect that our alanyl-glycine was contaminated with some glycine.

Among criteria commonly used in establishing the specificity of a new

analytical method is that in which measurements of the substance in question are made on multiples of the material to which the method is applied. The results of these determinations should be superimposable on those obtained on multiples of the pure substance when they are plotted on the same scale. The analyses of multiples of blood filtrate and urine (Fig. 3) coincide almost perfectly with determinations on a solution of pure glycine.

TABLE III  
*Recovery of Glycine Added to Blood and Urine*

	Glycine added*	Recovery of added glycine
	$\gamma$ per ml.	per cent
Blood	20	104
		94
	40	99
		86
	40	93
	50	91
	50	97
	50	105
	20	104
	50	87
Urine	20	89
	40	95
	100	96
	100	89
	200	94

\* The concentration of glycine in blood and urine was 20 to 30  $\gamma$  and 150 to 240  $\gamma$  per ml. respectively.

*Duplicability of Method and Recovery of Added Glycine (Tables I to III)*—The largest discrepancy between duplicate determinations of glycine was about 16 per cent. Most of the discrepancies fell within 6 per cent and the average deviation was 4 per cent. It was possible to recover 86 to 105 per cent of glycine added to blood or urine in amounts comparable to those originally present.

#### *Comment*

If the formaldehyde produced by the action of ninhydrin on the glycine in a protein-free filtrate of blood is not removed rapidly, substantial losses of formaldehyde occur. This may be due to interaction between formaldehyde and other amino acids which may not have been as rapidly converted as glycine into their respective aldehydes. It has been shown (14) that

formaldehyde exhibits a great affinity for certain amino acids, particularly those with SH or OH groups in the  $\beta$  position. Losses are greater at pH 4 and 7 than at pH 5.5. Our experience with glycine determinations at these pH levels is in accord with these findings.

The addition of more water during the distillation is necessary to assure complete recovery of formaldehyde. In dilute solution this substance exhibits very low volatility. This property is the basis for methods of separating other aldehydes by aeration (1, 14) from formaldehyde.

In order to avoid losses which may arise in the distillation of volatile aldehydes, the distillate usually is collected into bisulfite. Because of the low volatility of formaldehyde in very dilute solution, it can be distilled without loss when bisulfite is omitted. Since this reagent interferes markedly with the development of color, its omission is advantageous.

Certain facts concerning the reaction between chromotropic acid and formaldehyde are noteworthy. Eegriwe (10) and Boyd and Logan (11) state that strong sulfuric acid and heat are necessary to develop the color. They run the reaction in about 75 per cent sulfuric acid and heat for 10 minutes. Eegriwe (10) can detect as little as 1 part of formaldehyde in 250,000. Boyd and Logan (11) can measure about 50  $\gamma$  of formaldehyde in 17 ml. of aqueous distillate (3 parts in 1 million).

We have found that within certain limits the concentration of sulfuric acid determines the rate of the reaction between chromotropic acid and formaldehyde. When the ratio of acid to formaldehyde solution is 6:1, the color appears almost immediately; with a ratio of 2:1 the time required to complete the reaction is 10 minutes. 30 minutes are necessary when the ratio is 4:5, and in proportions of 3:5 more than 1 hour is necessary. The intensity of color, however, is not affected.

By using a ratio of 4 of acid to 5 of aqueous formaldehyde solution, the sensitivity of the method is increased over that of Eegriwe (10) and Boyd and Logan (11), since larger amounts of formaldehyde may be measured in a relatively smaller total volume of reaction mixture. In this way and by using an Evelyn photometer one can measure as little as 0.04  $\gamma$  of formaldehyde per ml. (1 part in 25 million).

Efforts to increase the sensitivity by using fuming, instead of concentrated, sulfuric acid or by adding inorganic salts of sodium, potassium, iron, nickel, copper, or zinc were unsuccessful.

#### SUMMARY

1. A method for the colorimetric determination of glycine in blood and urine is described.

2. The method is also applicable to the determination of formaldehyde.

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# A NEW REAGENT FOR THE DETERMINATION OF SUGARS

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For the determination of sugars several satisfactory methods are available, which are based on the oxidation of sugars either by cupric or ferric compounds in alkaline solutions. Our preference is for copper reagents mainly because they oxidize sugars more selectively than iron (ferricyanide) solutions. This is well illustrated by the fact that in blood filtrates deproteinized by the Folin-Wu method substances other than sugar reduce from 2 to 4 times as much iron as copper (1). On the other hand, ferricyanide possesses the virtue that, in contrast to cuprous oxide, it is not reoxidized by atmospheric oxygen once it is reduced to ferrocyanide. It is possible, however, to protect cuprous oxide against reoxidation by saturating the reagent solutions with sodium sulfate. By preventing reoxidation in this manner, it had become possible to determine with copper reagents as little as 0.01 mg. of glucose or any other sugar of an equal reducing power (2).

A detailed study of the Shaffer-Hartmann type of alkaline copper solutions (3) yielded considerable information in regard to the effect of the several constituents of these solutions upon the reduction equivalents of sugars. In the main it was shown that the lower the alkalinity, the higher the reduction values obtained; hence smaller quantities of sugars can be determined. Diminishing alkalinity, however, also entails decrease in the rate of oxidation and hence prolongation of the time required for heating the reaction mixtures. This is a source of difficulty when sugars are to be determined which react more slowly than glucose. In consequence, a "high alkalinity" copper reagent had to be prepared for the determination of maltose and other slowly reacting sugars.

Another finding of practical interest concerns the effect of potassium iodide, an ingredient of the Shaffer-Hartmann reagent. It was found that potassium iodide renders a small part of the cuprous ions soluble, thereby increasing the surface exposed to reoxidation and limiting the sensitivity of the reagents in the low ranges of sugar concentrations. The presence of iodide also makes these reagents unsuitable for colorimetric work. In the absence of potassium iodide, on the other hand, the reagents are unstable and are subject to self-reduction; so that cuprous oxide continually settles out at room temperatures, especially when exposed to sunlight. The instability also manifests itself in the poor reproduc-

bility of analytical results at the lower sugar concentrations. If tartrate is replaced by citrate in the reagent, this instability is completely eliminated; this advantage, however, is nullified by the fact that citrate causes a great decrease in the reducing power of sugars, so that the reagent becomes unsuitable for microanalysis.

### *New Reagent*

On account of these facts we had to use in our laboratory four varieties of alkaline copper-tartrate reagents, each serving a specific purpose. Recently we devised a reagent which unites the advantages of all four. It is sufficiently alkaline to allow the determination of maltose and other slowly reacting sugars; it contains no potassium iodide and yet is stable and shows no self-reduction at room temperatures even when exposed to direct sunlight; its high sulfate content and the absence of iodide virtually rule out the reoxidation of cuprous oxide. The absence of iodide also makes the reagent available for colorimetric work. In addition, the useful range of the reagent is greatly extended, so that a single reagent can be used for the determination of as little as 0.01 mg. and as much as 3.0 mg. of glucose or other sugars of equivalent reducing power. The results are gratifyingly consistent and reproducible even at the very lowest sugar concentrations.

The principal departure in the new reagent from the old is that the carbonate-bicarbonate buffer mixture was replaced by a phosphate buffer solution, consisting of approximately 0.1 M dibasic and 0.1 M tribasic sodium phosphate. Since it is known that any change in the concentration of any constituent of alkaline copper solutions influences the reaction with sugars (3), we have systematically examined the effect of varying concentrations of copper, of tartrate, and of phosphate. The concentrations finally settled upon represent the most favorable conditions as regards buffer action, stability, and sensitivity of the reagent.

*Constituents of Reagent*—1 liter of the solution contains 28 gm. of anhydrous disodium phosphate, 100 cc. of normal sodium hydroxide, 40 gm. of Rochelle salt, 8 gm. of cupric sulfate (crystalline), and 180 gm. of anhydrous sodium sulfate.

*Preparation*—The phosphate and tartrate are dissolved in about 700 cc. of water, the sodium hydroxide is added, and then, with stirring, 80 cc. of a 10 per cent copper sulfate solution are introduced. Finally the sodium sulfate is added and, when dissolved, the solution is diluted to 1 liter and allowed to stand for a day or two, during which time impurities separate out. The clear top part of the solution is decanted and the remainder filtered through a good grade of filter paper. As stated before, ~~this reagent~~ keeps indefinitely with no sign of deterioration.

### *Colorimetric Technique*

The iodometric determination of reduced copper is undoubtedly superior to the colorimetric technique, but only so far as visual (Duboscq type) colorimeters are concerned, and so long as the density of the color to be measured is relatively unstable. The photoelectric measurement of color density and the development of an improved color-producing reagent by Nelson (4), however, have eliminated the imperfections of the colorimetric technique when it is to be employed for microanalysis. In an adaptation of our micro reagent to photometric work, Nelson even extended the useful range of the reagent down to the determination of 5  $\gamma$  of glucose, whereas with the iodometric technique 10  $\gamma$  are about the limit. The improvement was attained by means of a new arsenomolybdate reagent devised by Nelson, which produces a color of higher intensity and greater stability than the older phosphomolybdate reagents. The use of Nelson's reagent in combination with the new copper-phosphate reagent is a further improvement of the colorimetric technique.

*Analytical Procedure.* In a 16  $\times$  150 mm. test-tube 2 cc. of the reagent and 2 cc. of sugar solution are mixed; the test-tube is covered with a glass bulb (dime store "marbles" are satisfactory), immersed in a boiling water bath, and heated for 10 minutes. After cooling, Nelson's chromogenic reagent is added, and the colored solution is diluted to a definite volume, from 10 to 25 cc., marked on the test-tube. The degree of dilution depends on the density of color. This colorimetric technique is as a matter of fact serviceable only as a micromethod, if, as it should be, extensive dilutions are to be avoided. Another limitation of the colorimetric technique is that it can be used only when the sugar solution is perfectly transparent and colorless. Thus, for example, for the determination of the reducing power of the milky or opalescent diastatic cleavage products of starch the colorimetric technique is unworkable.

### *Iodometric Technique*

For the iodometric measurement of the reduced copper the analysis may be started in the same manner as for the colorimetric technique; that is to say, as a general rule a measured volume of sugar solution is heated with an *equal volume* of the copper-phosphate solution. After cooling, a precisely measured amount of a standard potassium iodate solution and an appropriate amount of potassium iodide are added, the mixture is acidified, and, after complete oxidation of the cuprous copper, the free iodine is titrated with a thiosulfate solution. The procedure is somewhat simplified by incorporation of the potassium iodate in the copper solution. If 25 cc. of normal iodate are included in 1 liter of reagent, it is suitable for

the determination of any glucose solution containing from 0.2 to 60.0 mg. of the sugar per 100 cc.; i.e., from 0.01 to 3.0 mg. per 5 cc. of solution. This is a suitable reagent when the amount of glucose is between 2.0 and 3.0 mg. per 5 cc. of solution, but would lead to long, time-consuming titrations with lower glucose concentrations. For this reason it is best to prepare the copper reagent without the inclusion of iodate, and to add the latter to an aliquot of the basic solution only in such quantities as the anticipated range of glucose concentrations requires. Thus if the expected maximum concentration is not above 0.5 mg. per 5 cc. of solution, only about 5 cc. of normal iodate should be added to 1 liter of the reagent, and 10 cc. of iodate will be adequate if the amount of glucose does not exceed 1.0 mg. per 5 cc. of solution.

✓ The analytical procedure is carried out as follows: 5 cc. of the reagent and 5 cc. of the sugar solution are mixed in a 25 × 200 mm. Pyrex test-tube, covered with a glass bulb, and heated by immersion in a vigorously boiling water bath. The length of time required differs for different sugars. After cooling, potassium iodide is added. The amount of the iodide must be commensurate with the amount of the iodate. When the reagent contains 5 cc. of normal iodate per liter, 0.5 cc. of 2.5 per cent iodide suffices; when the iodate is 10 cc. per liter, 1 cc. of the iodide solution is added; for higher iodate content, 2 cc. of iodide solution are required. The potassium iodide solution may be prepared in advance; if alkalized with a knife tip of sodium carbonate, it keeps for a long time without decomposition. The iodide solution is added by running it from a pipette down the wall of the test-tube, without stirring or agitation. Following this, about 1.5 cc. of approximately 2.0 N sulfuric acid are added; the acid is rapidly dropped, rather than permitted to flow into the test-tube, with simultaneous agitation, so that the entire contents of the tube are mixed and acidified at once. A pipette with a wide opening (cracked-off tip) serves the purpose. For titration 0.005 N thiosulfate is used. This is prepared from time to time by dilution from a 0.1 N stock solution. When making the dilution, we add about 2 cc. of 10 per cent sodium hydroxide to 1 liter of solution for protection against atmospheric carbon dioxide.

*Rate of Oxidation of Glucose*—As shown in Fig. 1., the oxidation of glucose with the new reagent is quite rapid; when 5 cc. of a glucose solution are heated with 5 cc. of the reagent in a boiling water bath, in 3 minutes 67 per cent and in 5 minutes 97 per cent of the oxidation is accomplished, and in 8 minutes the reaction is complete. 10 minutes time as the standard heating period leaves a liberal margin of safety. For comparison, in Fig. 1 is included the reaction rate curve of Reagent 50 of Shaffer and Somogyi, in which 25 gm. of sodium carbonate and 20 gm. of bicarbonate

constitute the alkali. It may be noted that with this reagent it takes 16 minutes to attain about the same stage of the reaction as is reached with the new reagent in 8 minutes. When 18 per cent of sodium sulfate is incorporated in Reagent 50, in order to make it more comparable with the new reagent, the rate of reaction suffers a further decrease, so that it takes 24 minutes to reach the same stage which is reached by the other two reagents in 8 and 16 minutes, respectively. As the alkalinity of the reagent decreases, and the reaction slows down, the amount of copper reduced by sugars increases (in the carbonate-bicarbonate reagent, which contains sodium sulfate, prevention of reoxidation accounts for part of the increase in the reduction value). This advantage of low alkalinity is sacrificed by increasing the alkalinity, but this is amply compensated by

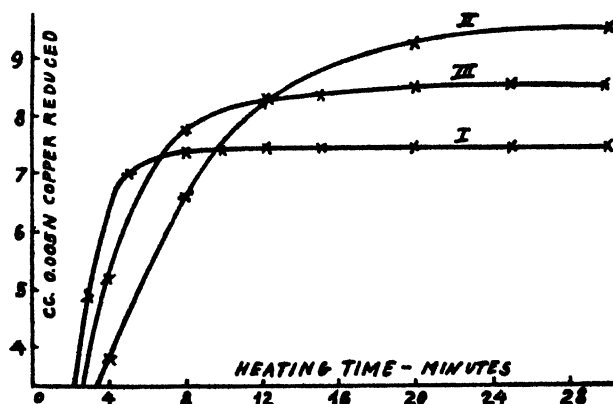


FIG. 1. Rate of copper reduction by glucose with three different reagents. Curve I, new reagent; Curve II, Reagent 50 of Shaffer and Somogyi with addition of 18 per cent sodium sulfate; Curve III, Reagent 50, unaltered.

shorter reaction time and, what is more important, by improvement in the consistent reproducibility of the results.

*Reduction Equivalent of Glucose*—Different quantities of glucose, in 5 cc. of solution, were heated with 5 cc. of the reagent for 10 minutes in a boiling water bath. A pair of blanks (5 cc. of water with 5 cc. of reagent) was heated with each batch. Titration was carried out as described before. The titration values, listed in Table I, are the differences between titration of blank and titration of the reagent-sugar mixtures. These figures are equivalent to cc. of 0.005 N copper reduced by glucose.

As may be noted, direct proportionality prevails between the amounts of glucose and the amounts of copper reduced. The proportionality is perfect within the limits of 0.05 and 3.0 mg. of glucose, but below 0.05

mg. the reduction equivalents tend to increase to an insignificantly slight degree. The equivalent of 1.0 mg. of glucose is 7.40 cc.; hence *each cc. of the titration value corresponds to  $1.0/7.4 = 0.135$  mg. of glucose.* Multiplication of this factor by the titration value gives the amount of glucose that was present in 5 cc. of solution.

TABLE I  
*Reduction Equivalents of Various Amounts of Glucose*

Glucose	0.005 N copper
mg.	cc
0.01	0.09
0.05	0.38
0.10	0.74
0.20	1.85
0.50	3.70
1.00	7.40 ✓
2.00	14.80
3.00	22.20

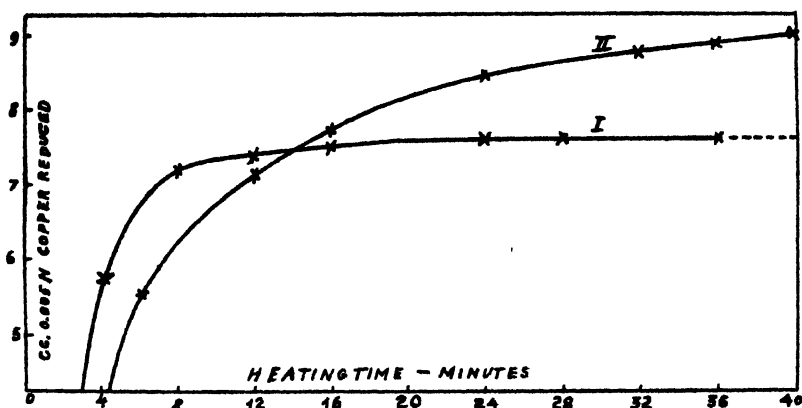


FIG. 2. Rate of copper reduction by maltose with new reagent (Curve I) and with Reagent 50 of Shaffer and Somogyi (Curve II).

*Rate of Oxidation of Maltose*—The oxidation of maltose in alkaline copper solutions proceeds at a considerably lower rate than the oxidation of glucose; so that completion of the reaction in the "carbonate-bicarbonate copper reagent, which was devised for glucose, is excessively drawn out. This was the reason that we had to introduce an additional "high alkalinity" carbonate copper reagent for studies involving maltose determination (5). The new phosphate-copper reagent renders such a special reagent

superfluous, for, as may be seen in Fig. 2, it oxidizes 97 per cent of the maltose in 12 minutes, and in less than 20 minutes the reaction is complete. Curve II in Fig. 2 shows the rate of reaction of maltose with Reagent 50, of Shaffer and Somogyi, to which the same amount of sodium sulfate had been added as to the new reagent. As may be seen, the oxidation of maltose with this reagent is still rather far from completion after 40 minutes of heating.

*Reduction Equivalent of Maltose*—We have determined with the new reagent the reducing power of different amounts of pure maltose. Pfanstiehl's pure maltose was used, with the results given in Table II.

TABLE II  
*Reduction Equivalents of Various Amounts of Maltose*

Maltose	0.005 N copper
mg.	cc.
0.025	0.09
0.05	0.20
0.125	0.49
0.25	0.95
0.50	1.92
1.00	3.85
2.00	7.71
4.00	15.47

These figures show a direct proportionality between the quantities of maltose and copper reduced, just as in the instance of glucose; 1 cc. of 0.005 N thiosulfate corresponds to 0.26 mg. of maltose, when the analysis is executed by the technique standardized for glucose, with the one difference that the heating period is 20 minutes instead of 10.

*Other Sugars*—As had been shown (3), some sugars, such as galactose and arabinose, require the same heating time as does maltose. There are, however, other sugars, as for instance lactose and mannose, the reaction rate of which is even lower. When the new reagent is to be employed for the determination of the latter, a reaction rate curve must first be constructed in order to find the appropriate heating time.

#### SUMMARY

A new alkaline copper reagent for the determination of sugars is presented. The alkali in this reagent is an equimolecular mixture of dibasic and tribasic sodium phosphate. The solution contains sulfate for the protection of cuprous oxide against reoxidation by atmospheric oxygen.

The reagent is suitable for both the iodometric and the colorimetric



technique and permits the accurate determination of 0.01 to 3.0 mg. of glucose or of 0.03 to 6.0 mg. of maltose by either technique.

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# DETERMINATION OF BLOOD SUGAR

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Several methods are now available by which blood sugar can be determined with such precision that the results are reproducible within 1 to 2 mg. per cent. Thus, it seems that there is scarcely any room for improvement in this respect. Yet our new copper-phosphate-tartrate reagent (1) offers definite advantages in the determination of blood sugar in that (a) it is equally suitable for the iodometric and the colorimetric determination of cuprous oxide, (b) a single reagent is suitable for macro- and micro-analysis, its useful range extending from 0.01 to 3.0 mg. of glucose, (c) in the lowest ranges of sugar concentrations the new reagent yields more consistent and more closely reproducible results than our copper-carbonate-tartrate micro reagent.

Another change in blood analysis, that we have introduced in our laboratory, concerns the deproteinization of blood. To remove non-sugar reducing substances along with the proteins, we still find zinc sulfate and alkali satisfactory; comparative examinations convinced us that replacement of zinc by cadmium carries no advantage whatsoever. We have found, on the other hand, that substitution of barium hydroxide for sodium hydroxide improves the method and expands its usefulness. One advantage of this modification is that it renders superfluous a separate deproteinization method for plasma and serum. Zinc sulfate with sodium hydroxide is good only for whole blood, but fails to deproteinize plasma completely; hence for the latter purpose we had to use copper sulfate in combination with sodium tungstate. With barium hydroxide as the alkali, the same reagents which are prepared for the deproteinization of whole blood also are adequate for plasma and serum. We attribute this extension of usefulness to the considerable adsorptive capacity of the barium sulfate that is formed in the reaction. This quality has made the zinc-barium precipitation a serviceable method in our laboratory for the purification of urine, of muscle and liver extracts, etc.

A second advantage of the use of barium hydroxide is that it introduces no salts into the blood filtrate or other tissue extracts, a desirable feature in several types of studies, as for instance when the extracts must be evaporated to small volumes. A third service rendered by barium hydroxide is that it precipitates anticoagulants, such as fluoride and oxalate,

which, if present in solution in too large quantities, as is not infrequently the case in clinical blood samples, may interfere with deproteinization.

*Reagents*-- 5.0 per cent solution of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.3 N barium hydroxide. The accuracy of these concentrations is less important than the requirement that the alkali must neutralize the zinc sulfate solution precisely, volume for volume, when titration is performed with phenolphthalein as indicator. To carry out the titration, 10 cc. of the zinc sulfate solution are introduced into a flask, diluted with about 100 cc. of water, and then the alkali is run in dropwise, under continual agitation, until the phenolphthalein turns pink and the color persists for at least 1 minute. (Rapid titration, with the alkali running in, gives false endpoints.) On the basis of the titration the solution that is more concentrated is diluted to match the other.

TABLE I

*Deproteinization of Blood, Plasma, or Serum at Different Dilutions, with Zinc Sulfate and Barium (or Sodium) Hydroxide*

Procedure No	Water	Blood	0.3 N alkali	5 per cent $\text{ZnSO}_4$	Total volume	Dilution
	cc	cc	cc	cc	cc	
1	5.0	1.0	2.0	2.0	10.0	1:10
2	7.5	0.5	1.0	1.0	10.0	1:20
3	7.0	0.2	0.4	0.4	8.0	1:40
4	7.5	0.1	0.2	0.2	8.0	1:80
5	3.0	0.2	0.4	0.4	4.0	1:40
6	3.5	0.1	0.2	0.2	4.0	1:80

The concentrations of these solutions are so chosen as to make their use flexible; *i.e.*, the same solutions should be suited for deproteinization of large as well as small amounts of blood, down to 0.1 cc. In Table I are presented six variants of the deproteinization process, adapted to various amounts and dilutions of the blood; it should be obvious, however, that these are not stereotyped recipes, so that they may be altered to fit any particular condition.

For deproteinization, the blood is laked in a measured amount of water, and the alkali is admixed, 2 volumes for each volume of blood; this is followed by 2 volumes of the zinc sulfate solution, and then the mixture is vigorously shaken and filtered. If the two reagents are correctly balanced, precipitation is perfect as manifested by the absence of foaming on shaking and by the rapidity of filtration. The sugar in the blood filtrates can be determined with the new reagent either by the iodometric or the colorimetric method, as described in the preceding paper (1).

*Iodometric Method*

For maximum accuracy 5 cc. of the reagent are to be used with 5 cc. of 1:10 blood filtrate, the latter being prepared as directed under Procedure 1, in Table I. For microdeterminations in 0.2 or 0.1 cc. of blood the proportions of water, blood, and deproteinizing reagents are given under Procedures 3 and 4, respectively, in Table I; in either case 5 cc. of the filtrate are to be used for analysis in the same manner as in the instance of 1:10 filtrates.

The lowest blood sugar concentration that can be determined by this method is 2.0 mg. per 100 cc. of blood, if 5 cc. of 1:10 filtrate, containing 0.01 mg. of glucose, are used for analysis. If the analysis is carried out with only 2 cc. of 1:10 filtrate (and 2 cc. of reagent), then 5 mg. per cent are the lowest blood sugar that can be determined. In the micromethod 8 mg. per cent are the lower limit when 0.2 cc. of blood (5 cc. of 1:40 filtrate) is used, and 16 mg. per cent when only 0.1 cc. of blood (5 cc. of 1:80 filtrate) is available.

Calculation of the glucose content from the titration value (denoted hereafter as  $T$ ) is simple, since each cc. of titration value corresponds to 0.135 mg. of glucose (1). Thus when 5 cc. of 1:10 filtrate (corresponding to 0.5 cc. of blood) are used for analysis, the blood sugar equals  $0.135T \times (100/0.5)$  or in simplified form, *blood sugar* =  $27 \times T$  mg. per 100 cc. In the micromethod with 0.2 cc. of blood, *blood sugar* =  $108 \times T$  mg. per 100 cc., and with 0.1 cc. of blood, *blood sugar* =  $216 \times T$  mg. per cent. When 2 cc. of 1:10 blood filtrate are used with 2 cc. of the reagent, *blood sugar* =  $67.5T$  mg. per cent.

Inclusion of potassium iodide in the reagent, as is known, upsets the proportionality between the amounts of sugar and of copper reduced and, in addition, curtails the usefulness of the reagent at the lowest range of sugar concentrations. These two drawbacks may be sufficiently outweighed under certain circumstances by the economy in time and work that can be attained by incorporating iodide in the reagent, as in the Shaffer-Hartmann type of reagents. A case in point is the situation in clinical laboratories where large numbers of blood sugar determinations are to be performed day after day.

For this purpose 8 gm. of potassium iodide and 25 cc. of normal potassium iodate are added to 1 liter of the reagent that had been prepared for general use. For analysis 2 cc. of a 1:10 blood filtrate are mixed with 2 cc. of the reagent in an 18  $\times$  150 mm. test-tube, covered, and heated for 12 minutes. After cooling, 1 cc. of approximately 2.0 N sulfuric acid is rapidly added, with agitation and, after complete clarification of the solution, titration with 0.005 N thiosulfate is carried out. An accurate 10 cc. burette with 0.05 cc. divisions should be used.

The titration can be, and should be, carried out with accuracy, since the end-point turns with less than 0.01 cc. of 0.005 N thiosulfate. If measurements of the copper reagent and of the blood filtrate and those involved in the deproteinization procedure are performed with adequate care, the values obtained for blood sugar are duplicable within 1 mg. per cent. The lowest blood sugar that can be reliably determined with this technique is 10 mg. per cent, the highest 600 mg. per cent. Since no direct proportionality obtains between reduction values and sugar, the reduction equivalents had to be experimentally determined; they are given in Table II.

TABLE II

*Glucose per 100 Cc. of Blood, Corresponding to Titration Values When 2 Cc. of 1:10 Blood Filtrate and 2 Cc. of Copper Reagent\* Are Heated in Water Bath for 10 Minutes*

0.005 N thiosul- fate	0.005 N sodium thiosulfate									
	0	0.1 cc.	0.2 cc.	0.3 cc.	0.4 cc.	0.5 cc.	0.6 cc.	0.7 cc.	0.8 cc.	0.9 cc.
Glucose in 100 cc. of blood or plasma										
cc	mc	mg.	mc	mg.	mc	mg.	mg.	mg.	mg.	mc
0	0	11	18	25	32	40	47	54	61	68
1	75	82	89	96	103	110	117	124	131	137
2	143	149	155	162	169	175	182	189	195	202
3	209	215	221	228	234	241	248	254	261	268
4	275	281	288	295	302	309	315	322	329	336
5	342	349	356	363	370	376	383	389	396	403
6	409	416	422	429	436	443	449	456	462	469
7	476	482	489	496	503	510	516	523	529	536
8	543	549	556	563	570	577	583	590	596	603

\* Contains 25 cc. of normal  $\text{KIO}_3$  and 8 gm. of KI per liter.

### *Colorimetric Method*

For the colorimetric procedure the basic form of the copper reagent is used which contains neither iodate nor iodide. As the color-producing reagent Nelson's arsenomolybdate solution (2) should be used. Since the amount of glucose in this procedure must not be very high, only 2 cc. of 1:20 blood filtrate are needed for analysis, prepared according to directions given for Procedure 2, in Table I.

As in the iodometric procedure, the filtrate is mixed in an 18 × 150 mm. test-tube with 2 cc. of the copper reagent, and the tube is covered with a glass bulb and heated in a boiling water bath for 10 minutes. There is no need for special tubes with restricted necks (Folin-Wu tubes), since reoxidation of cuprous oxide with the new reagent is practically nil in

ordinary test-tubes. After cooling, Nelson's reagent is added and the solution is diluted to 25 cc., marked on the test-tube. For microanalysis with 0.2 or 0.1 cc. of blood, deproteinization is carried out according to directions given in Procedures 5 and 6 (Table I), respectively. The further procedure is the same as with 1:20 blood filtrates, but the colored solution is diluted to 10 instead of 25 cc. As to the preparation of the arsenomolybdate reagent, and for any further details concerning the colorimetric method, the reader is referred to Nelson's original directions (2).

#### SUMMARY

1. The author's method of blood deproteinization by zinc sulfate and sodium hydroxide was modified by substituting barium hydroxide for sodium hydroxide. Advantages of the modification are outlined.

2. Iodometric and colorimetric determination of blood sugar with a new copper-phosphate-tartrate reagent is described.

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# THE FREE CHEMICAL GROUPS OF TYROCIDINE

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If tyrocidine is assumed to be a peptide of eleven amino acids,<sup>1</sup> including among other residues 1 residue each of tyrosine and ornithine, 2 residues each of dicarboxylic acids and of labile ammonia (1-3), and if these amino acids are joined into a peptide chain by linkages between the  $\alpha$ -amino and carboxyl groups, then one might predict that the following free groups will exist in the molecule: (1) a terminal amino (or imino) group; (2) a terminal carboxyl group; (3) a side chain amino group due to the  $\delta$ -amino group of ornithine; (4) two side chain carboxyl groups, due to the distal carboxyl groups of the dicarboxylic acids, bound to the ammonia residues as amides; and (5) a phenolic hydroxyl group.

On the other hand, there are obviously many other ways in which the known residues might be joined together to produce a different collection of free groups. In this investigation, evidence was obtained for the existence of a free amino group due to the  $\delta$ -amino group of ornithine, for the free existence of the phenolic group, and for the absence of detectable quantities of any other free amino or imino groups. This communication does not add to the information about the nature of the free carboxyl groups or of the labile ammonia.

A general statement of the results which have been obtained with the procedures employed follows.

*On Amino Groups*—S-Methylisothiurea (4, 5) reacted in a few hours with tyrocidine in methanol at room temperature, to convert the *l*(+)-ornithine residue practically quantitatively to an *l*(+)-arginine residue, without producing detectable quantities of other alkyl guanidinium compounds. After hydrolysis by acid the treated tyrocidine revealed one arginine residue per 13 tyrocidine nitrogen atoms, the amount found being the same by colorimetric determination based upon the Sakaguchi reaction (6) and by determination by liver arginase. The Sakaguchi reaction was abolished by the action of arginase.

*On Amino and Phenolic Groups*—*p*-Toluenesulfonyl chloride reacted with tyrocidine in pyridine solution quickly to introduce labile *p*-toluenesulfonyl groups and more gradually to introduce alkali-stable groups.

<sup>1</sup> Hotchkiss (1) has advanced evidence for considering the molecule to be about twice this size.



The derivatives formed by the action of aromatic sulfonyl chlorides with amino and imino groups are generally more resistant to acid hydrolysis than peptide links, and are highly resistant to cleavage by alkali. This stability has been utilized for the study of the free groups of proteins (*cf.* Gurin and Clarke (7)). About 1 gm. atom of sulfur per 4 gm. atoms of nitrogen (exclusive of ammonia nitrogen) could be introduced into tyrocidine. The sulfonyl derivatives surviving acid hydrolysis appeared to include *O-p*-toluenesulfonyl-*l*(-)-tyrosine and  $\delta$ -*p*-toluenesulfonyl-*l*(+)-ornithine. The crystallization of the latter succeeded in only one case and in poor yield. *N-p*-Toluenesulfonyl derivatives of monoamino acids, or of aspartic acid, glutamic acid, proline, or ammonia could not be detected.

*On Phenolic Groups*—Methylation of tyrocidine with methyl sulfate appeared to produce complete methylation of the phenolic hydroxyl group, but no N methylation could be detected.

The following were prepared as reference substances:  $\delta$ -*p*-toluenesulfonyl-*l*(+)-ornithine, m.p. 210–215°, the  $\alpha$ -benzoyl and  $\alpha$ -acetyl derivatives of this, m.p. 183° and 153° respectively, di-*p*-phenylphenacyl esters of *N-p*-toluenesulfonyl-*l*(+)-aspartic acid and *N-p*-toluenesulfonyl-*l*(+)-glutamic acid, both melting at 138–141°.

A catalytic effect of *p*-toluenesulfonic acid, and to a smaller degree of 2-naphthalenesulfonic acid, upon the acid hydrolysis of tyrocidine was incidentally observed. Introduction of alkali-stable *p*-toluenesulfonyl groups into tyrocidine, on the other hand, retarded the acid hydrolysis of tyrocidine. No catalysis of the hydrolysis of gramicidin by *p*-toluenesulfonic acid was observed.

#### EXPERIMENTAL

*Action of Methylisothiurea upon Tyrocidine*—500 mg. of S-methylisothiurea sulfate were suspended in methanol and 2 milliequivalents of sodium methylate in methanol added at 0°. After thorough agitation the precipitate was removed and the solution added to 100 mg. of tyrocidine hydrochloride<sup>2</sup> in 10 ml. of methanol. After 24 hours at room temperature the solvent was concentrated to 1 ml. and added to 10 ml. of dilute aqueous hydrochloric acid. The precipitate was washed repeatedly with dilute hydrochloric acid, and hydrolyzed as usual for 24 hours.

The hydrolysate appeared to contain about 5 mg. of alkyl guanidinium compounds expressed as arginine, by the Sakaguchi reaction applied as described by Jorpes and Thoren (6). After tyrosine was removed, the apparent arginine by this reaction increased to 1 arginine per 13 original tyrocidine nitrogen atoms, exclusive of ammonia. The interference of

<sup>2</sup> The tyrothricin from which the tyrocidine hydrochloride was isolated was supplied through the generosity of Mr. Leo Wallerstein of the Wallerstein Company, Inc.

tyrosine with the color reaction was demonstrated by Weber (8). An impure preparation of arginase was made from a mouse liver by the use of acetone, as described by Richards and Hellerman (9). Arginine was determined by the urea released by arginase, by measuring both the carbon dioxide (Van Slyke (10)) and the ammonia released. The arginine found represented 1 molecule per 12.6 to 13.2 original tyrocidine nitrogen atoms, exclusive of ammonia, in three preparations of guanylated tyrocidine. Arginase abolished to the extent of 99 per cent the Sakaguchi reaction of the hydrolysates. Added arginine could be recovered in these arginase-containing solutions by the Sakaguchi reaction. Our average value for ornithine (by phosphotungstic acid) in tyrocidine was 1 ornithine per 12.2 tyrocidine nitrogen atoms, exclusive of ammonia. Analyses for total and amino nitrogen of the insoluble phosphotungstates separated from the hydrolysates failed to reveal the presence of ornithine. Arginine was isolated and identified as the benzilidine derivative (11).

*Benzoylation of Tyrocidine*—50 mg. of tyrocidine were treated in 0.5 ml. of anhydrous pyridine with 0.13 ml. of benzoyl chloride with cooling. After 4 hours at room temperature, the product was precipitated by benzene, washed thoroughly with benzene and water, and dried at 100° over phosphorus pentoxide. Benzoyl groups were determined by titrating with alkali the benzene-soluble portion of an acid hydrolysate. The results showed one benzoyl group for 4.3 tyrocidine nitrogen atoms.

*Action of *p*-Toluenesulfonyl Chloride upon Tyrocidine*—300 mg. of tyrocidine hydrochloride in 6 ml. of pyridine were treated with 1.2 gm. of *p*-toluenesulfonyl chloride and left 72 hours at room temperature. The preparation was precipitated, washed, and dried, as described for benzoylated tyrocidine. The product contained 10.0 per cent nitrogen. The ratio of non-ammonia N to sulfur was 4.0. 3 hours of acid hydrolysis (acetic acid plus 2 volumes of 6 N hydrochloric acid) yielded a ratio of amino N to total N of 0.51, 10 hours of hydrolysis, of 0.71 (ammonia removed in both cases), indicating a slower hydrolysis than that of untreated tyrocidine in the presence of *p*-toluenesulfonic acid. The product resulting from 10 hours of hydrolysis was taken to dryness, and dissolved in water. Solubility was complete and ether extraction removed only traces of nitrogen. The solution was extracted with ethyl acetate, and the extracted fraction neutralized in aqueous solution. The needles which separated melted, after recrystallization, at 189°. The product contained 4.20 per cent  $\alpha$ -amino nitrogen (12) compared with the value of 4.18 per cent calculated for O-toluenesulfonyl tyrosine. Treatment with *p*-toluenesulfonyl chloride yielded needles melting at 115°,<sup>3</sup> only slightly depressed upon admixture with O, N-ditoluenesulfonyl-*l*(-)-tyrosine (13).

<sup>3</sup> The melting points recorded have been corrected for the emergent thermometer stem.

Butanol extraction of the ethyl acetate-extracted hydrolysate, followed by neutralization in aqueous solution of the material extracted, yielded in one instance a small quantity of needles melting at 200–205°. Upon acetylation a product was obtained melting at 151°. The melting point in admixture with synthetic  $\alpha$ -acetyl- $\delta$ -*p*-toluenesulfonyl-*l*(+)-ornithine was 150°. Crystalline derivatives of toluenesulfonylornithine were not obtained in two other experiments.

A further sample of the tosylated tyrocidine was hydrolyzed in 3 *N* sodium hydroxide 48 hours at 100°. An aliquot of the hydrolysate was analyzed for *p*-toluenesulfonic acid by addition after acidification by hydrochloric acid of half its volume of an 8 per cent aqueous solution of 2-naphthylamine hydrochloride. The crystalline precipitate was washed and weighed. Upon recrystallization the salt melted at 217°. Slotta and Franks (14) have reported 218° for the 2-naphthylamine salt of *p*-toluenesulfonic acid. 61 per cent of the sulfur was precipitated by this reagent; correction for solubility indicated that about 66 per cent of the sulfur was present as free *p*-toluenesulfonic acid.

The alkaline hydrolysate contained no ether-soluble sulfur, and the distribution of sulfur between ethyl acetate and water was so low as to exclude the presence of significant amounts of *p*-toluenesulfonyl derivatives of the monoaminomonocarboxylic acids, of proline, of ammonia, and of glutamic acid. (The distribution coefficient of *p*-toluenesulfonyl-*l*(+)-glutamic acid between ethyl acetate and water was found to be about unity.) Study of a butanol extract of the aqueous residue from the ethyl acetate extraction failed to show evidence of any *p*-toluenesulfonylaspartic acid. No barium salts precipitable by ethanol could be found in this extract.

*Methylation of Tyrocidine*.—To a solution of 103 mg. of tyrocidine hydrochloride in 2 ml. of methanol and 0.05 ml. of 4 *N* sodium hydroxide, there were added, in thirteen portions during 1 hour, 1.6 ml. of 9 *N* sodium hydroxide and 0.85 ml. of dimethyl sulfate. After 16 hours at 37°, the solution was treated with 25 ml. of *N* hydrochloric acid, and the resulting precipitate was washed with hydrochloric acid. After being dried *in vacuo*, the product contained 12.8 per cent nitrogen. A portion was hydrolyzed as usual in acetic acid plus hydrochloric acid for 24 hours. Only traces of ammonia nitrogen were present. (The alkalinity employed in the methylation rapidly releases ammonia from tyrocidine even at room temperature.) The ratio, amino nitrogen to total nitrogen, in the hydrolysate was 0.84, whereas the value 0.86 was obtained for ammonia-free hydrolysates of tyrocidine. Thus, little if any *N* methylation was indicated.

The bases were precipitated from the hydrolysate by phosphodo-

decatungstic acid according to Van Slyke, Hiller, and Dillon (15) after removal of tryptophane. 16.3 per cent of the nitrogen of the hydrolysate was precipitated, and this nitrogen was all released in 20 minutes by nitrous acid. A very similar proportion of nitrogen was recovered in the same way from unmethylated tyrocidine. Thus, no N methylation of ornithine could be detected.

*O-Methyltyrosine*—The method of Folin and Marenzi (16) showed that 80 per cent of the tyrosine of the above hydrolysate was free. However, when hydrolysis was carried out in a mixture of 1 volume of acetic acid and 2 volumes of 4 N sulfuric acid for a period of 4 hours, catalyzed by *p*-toluenesulfonic acid, very little free tyrosine could be detected (*cf.* (17)). The hydrolysate was acetylated according to du Vigneaud and Meyer (18),

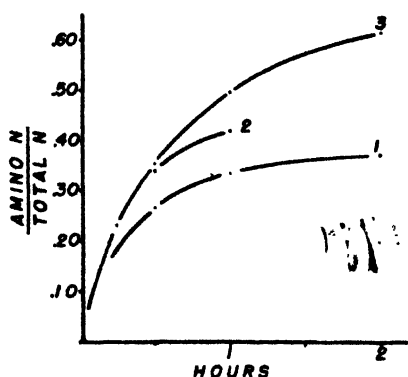


FIG. 1. Acceleration of the hydrolysis of tyrocidine in boiling acetic acid-hydrochloric acid mixtures by aromatic sulfonic acids. Curve 1, in the absence of sulfonic acids; Curve 2, in the presence of 2-naphthalenesulfonic acid; Curve 3, in the presence of *p*-toluenesulfonic acid. A ratio of amino N to total N of 0.81 represents the maximum obtained during 48 hours of acid hydrolysis of tyrocidine.

extracted with chloroform after acidification, and the extracted material was crystallized twice from water. The crystals were obtained in a yield representing 6 per cent of the nitrogen of tyrocidine. A neutralization equivalent of 204 (calculated for acetylmethyltyrosine, 209) and a melting point of 148° were obtained. This melting point was not depressed by admixture with synthetic N-acetyl-O-methyl-(–)-tyrosine.

*Catalysis of Hydrolysis of Tyrocidine*—If tyrocidine was treated in pyridine briefly with a small amount of *p*-toluenesulfonyl chloride, mainly alkali-labile groups were introduced. This product was found to undergo very rapid acid hydrolysis of its peptide links. This accelerated hydrolysis appeared to be due to catalysis by *p*-toluenesulfonic acid. 50 mg. of tyrocidine hydrochloride were hydrolyzed in a boiling mixture of 2 ml.

of acetic acid and 4 ml. of 6 N hydrochloric acid containing 17 mg. of *p*-toluenesulfonic acid. Similar experiments were performed with an equivalent concentration of 2-naphthalenesulfonic acid and in the absence of sulfonic acids. The comparative rates of hydrolysis are shown in Fig. 1. No catalysis of the hydrolysis of gramicidin by *p*-toluenesulfonic acid was detected.

*δ-p-Toluenesulfonyl-l-(+)-ornithine*--205 mg. of *l*(+)-ornithine dihydrochloride (Hoffmann-La Roche) were dissolved in 3 ml. of water and treated at 100° with an excess of copper carbonate. The solution was filtered and 2 ml. of N sodium hydroxide were added to the solution of the copper salt. 287 mg. of *p*-toluenesulfonyl chloride were added in four portions in ether solution at hourly intervals, each addition being accompanied by 0.75 ml. of N sodium hydroxide. Shaking was maintained meanwhile, and for a total of 5 hours. The solution was then acidified and copper removed with hydrogen sulfide, and the filtrate was concentrated and adjusted to about pH 7. The crystals separating were recrystallized from hot water, with a yield of 60 per cent of the theoretical. They were long slender needles melting at 210–215° with decomposition. The compound formed a slightly soluble phosphotungstate.

<i>Analysis</i>	$C_{12}H_{18}N_2O_4S$	Calculated.	N 10.37, amino N 5.18
		Found.	" 10.3 , " " 5.10

*α-Benzoyl-δ-p-toluenesulfonyl-l-(+)-ornithine* was prepared by benzoylation by the Baumann-Schotten technique, and crystallized from acetic acid. Needles melting at 183° were obtained in 60 per cent yield.

*α-Acetyl-δ-p-toluenesulfonyl-l-(+)-ornithine* was prepared by the action of acetic anhydride (18). The product was extracted, after acidification of the solution, by ethyl acetate, and crystallized from acetone solution by the addition of diethyl ether. Needles melting at 153° were obtained. This derivative was obtained pure more readily than the benzoyl derivative.

*p-Phenylphenacyl esters of p-toluenesulfonyl-l-(+)-glutamic acid and p-toluenesulfonyl-l-(+)-aspartic acid* were prepared by treating the tosylated amino acids (13) with *p*-phenylphenacyl bromide (19). After two crystallizations from absolute alcohol, both derivatives melted at 138–141°. This melting point was strongly depressed upon mixing the two derivatives. Nitrogen analyses were as follows: calculated 2.07, 2.03 per cent, respectively; found 2.10, 2.07 per cent.

#### DISCUSSION

The results obtained with methylisothiurea indicate that the  $\delta$ -amino group of ornithine exists free in tyrocidine, or in such a state that it reacts in the manner of a free amino group with this reagent at room temperature.

Whereas practically complete reaction occurred with the ornithine, no significant quantities of other substances yielding the Sakaguchi reaction were formed. Weber (8) has shown that this reaction is characteristic of monoalkyl guanidinium compounds. This result argues against the presence of any other free amino group besides that of ornithine. Surprisingly, the action of nitrous acid for 30 minutes upon acetic acid solutions of tyrocidine did not reduce the recovery of ornithine by phosphotungstic acid.

The number of benzoyl and *p*-toluenesulfonyl groups which could be introduced into tyrocidine (three for 11.6 and 12 nitrogen atoms respectively, exclusive of ammonia) corresponds fairly closely to the number of acetyl groups (three for 11 nitrogen atoms, exclusive of ammonia) which Hotchkiss introduced by the action of acetic anhydride (1). Two of these groups are probably accounted for by the distal amino group of ornithine and the phenolic group of tyrosine.

No explanation is advanced as to why evidence could not be obtained of a terminal amino group in tyrocidine. Calculations (1) suggest the absence of a free terminal carboxyl group as well. The sum of the amide nitrogen plus free carboxyl groups of tyrocidine found by Hotchkiss (2.05 per 13 total nitrogen atoms) corresponds closely with the analytical values for dicarboxylic amino acids (2.1 per 13 nitrogen atoms (1, 2)).<sup>4</sup> Thus there is no indication of a terminal carboxyl group in addition to the side chain groups presumably contributed by these acids. In this connection it may also be pointed out that no free amino or carboxyl group has been demonstrated in the gramicidin molecule.

#### SUMMARY

An examination of the free chemical groups of the tyrocidine molecule has been made by several techniques. The results indicate that the  $\delta$ -amino group of ornithine and the phenolic hydroxyl group of tyrosine are free in the polypeptide, since both were coupled to the *p*-toluenesulfonyl group by the action of *p*-toluenesulfonyl chloride in pyridine solution; the second was methylated by methyl sulfate, while the ornithine residue in tyrocidine was converted practically completely to an *l*(+)-arginine residue by the action of methylisothiourea. No evidence could be obtained for the presence of any *N*-*p*-toluenesulfonyl derivatives after hydrolysis by alkali except that of ornithine. No other guanidinium compounds

<sup>4</sup> The action of hypobromite according to the Hoffmann amine reaction was tested under several sets of conditions to ascertain whether the labile ammonia of tyrocidine was bound by the amide link to carboxyl groups and, if so, to what amino acid residues. Too much damage to the tyrocidine molecule resulted to accomplish this purpose.

except arginine could be detected after the action of methylisothiurea. Thus both procedures failed to reveal the presence of another free amino group in addition to the  $\delta$ -amino group of ornithine.

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# CHEMICAL AND PATHOLOGICAL CHANGES IN DIETARY CHLORIDE DEFICIENCY IN THE RAT

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Only in recent years has it been demonstrated unequivocally that elimination of chloride from the diet leads to an impairment of health. The authors reported in a previous paper (1) that chloride deficiency (0.012 per cent Cl in the diet) caused a reduction in the growth rate, a reduction in blood chloride, an increased blood bicarbonate, and almost a cessation of the urinary excretion of chloride.

Growth and the energy and protein metabolism in chloride deprivation were studied by Voris and Thacker (2) on pair-fed rats maintained on a diet containing 0.02 per cent chloride. The rats on the deficient diet showed a loss of appetite but an increased water consumption. The deficient rats gained less water (as per cent of total fat-free gain) and less fat than the controls. The ratio of water gained to protein gained was lowered, leading the authors to characterize the tissue cells as water-poor. More of the energy of the ingested food was lost as heat and less stored in the chloride-low rats than in the controls. The deficient animals gained more protein in terms of per cent of gain in body weight but gained less in per cent of total body protein. This was reflected by an increase in the output of urinary nitrogen and a decrease in nitrogen retention. The deficient animals showed a greater gain, proportionately, of residual (mainly inorganic) substance.

The effect of the chloride deficiency on the mineral composition of the body was more closely analyzed by Thacker (3) on the above rats. He found that there was a decrease in the content of chloride, sodium, and potassium, and an increase in the content of calcium, magnesium, and phosphorus, presumably because the ratio of soft tissue to skeleton is lowered. The concentrations of chloride, sodium, and potassium were also decreased with respect to the water content and to the water gained, but without disturbing the ratio of sodium to potassium. There was a decrease in the retention of all the above mineral elements except chloride.

The present investigation was undertaken to obtain more of an insight into the nature of the physiological disturbances caused by deficiency of chloride. Estimations of the extracellular phase were made from the chloride composition and from the distribution of the radioactive isotope,  $\text{Na}^{22}$ , in view of the apparent alteration in water metabolism and because



it seemed logical that the bodily stores of chloride might be made to go further by a reduction of the extracellular fluid volume.

Further evidence for the existence of a state of alkalosis (largely compensated) in this deficiency was sought from the excretion of citrate in the urine.

Chloride is the only one of the major mineral constituents of the body for which proof has been lacking that its deficiency in the diet leads to pathological manifestations. The gross appearance of the kidneys of the chloride-deficient rats suggested that histological examination might reveal abnormalities in this organ, and this inference proved to be correct. A dietary intake of chloride below the ability of the organism to conserve its bodily stores of this ion has been found to produce kidney damage.

### Methods

Some modification was made in the diet from that used in the previous investigation (1). The basal constituents were not altered but several changes were made in the salt mixture and the water-soluble vitamins were supplied from only crystalline sources.<sup>1</sup> All salts employed were recrystallized or purified by washing. The casein for the diet was washed with dilute acetic acid, followed by distilled water until halogen-free, then dried, and ground. The choline acetate was prepared from choline chloride by precipitating the chloride with lead acetate and removing the excess lead with hydrogen sulfide.

The new chloride-low diet was found to contain only 2 to 5 mg. of halide per 100 gm. of food, other than the added iodide, as contrasted with up to 12 mg. in the previous work. The chloride-deficient diet contained a total carbonate and bicarbonate content of 2 per cent, the control diet 0.9 per cent carbonate and 0.6 per cent chloride.

The rats employed in the experiments were transferred to screen-bottomed cages and placed on the synthetic rations at between 3 to 4 weeks of age and, except for those that were pair-fed, were allowed food and distilled water *ad libitum*. All cages were cleaned and rinsed with distilled water in advance to remove any possible chloride contamination.

Blood for chloride and  $\text{Na}^{22}$  analysis was collected and centrifuged under oil. The blood was obtained by heart puncture under amytal anesthesia and the animals were subsequently sacrificed for analysis of tissues. The chloride titrations were performed on tungstate filtrates of the serum according to the mercuric nitrate method of Schales and Schales (4).

<sup>1</sup> The salt mixture in gm. and the water-soluble vitamins in mg. per kilo of diet had the following composition:  $\text{CaCO}_3$ , 15;  $\text{NaCl}$ , 10 (control diet only);  $\text{NaHCO}_3$ , 15 (deficient diet only);  $\text{K}_2\text{HPO}_4$ , 20;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6; ferrous alum, 2;  $\text{KI}$ , 0.01;  $\text{MnSO}_4$ , 0.05;  $\text{CuSO}_4$ , 0.05;  $\text{ZnSO}_4$ , 0.001;  $\text{C}_6\text{C}_2\text{H}_3\text{O}_2$ , 0.001; thiamine hydrochloride, 8; ribofla-

Samples for tissue chloride analysis were dry-ashed at a low temperature in porcelain dishes after the addition of chloride-free sodium bicarbonate and the chloride was determined by the silver nitrate-potassium thiocyanate titration method of Whitehorn (5). The citric acid was determined by the colorimetric method of Pucher, Sherman, and Vickery (6), with 1,4-dioxane as a solvent as suggested by Johnson (7), instead of pyridine, on 8 to 16 hour urine samples collected from individual rats. The urine collections covered all 24 hours of the day.

In the collection of tissues, skeletal muscle samples were taken from the hind legs (chiefly thighs) with as much fat and nerve removed as possible. The capsules were removed from the kidney samples. The brain samples consisted of the cerebrum, cerebellum, and brain stem. The carcasses on which chloride determinations were performed were from the first series of rats placed on the modified deficient diet and these were less deficient than later ones. The carcasses used for determination of the sodium extracellular phase were from a later series in which the diet was lower in chloride. The carcass usually constituted the remains of the rat after the liver, kidney, skin samples, muscle samples, and as much blood as possible had been removed. In certain instances the other tissues listed in Tables I to III were also removed.

The water content of tissues and carcass was determined by drying at 90-100° in an electrically heated oven for 48 hours or longer.

The sodium partition was determined with the radioactive isotope  $\text{Na}^{22}$ , which has a half life of 3 years. This was prepared by bombarding magnesium metal with deuterons (8) in the medical cyclotron of the University of California Radiation Laboratory. The  $\text{Na}^{22}$  was administered as the chloride by intraperitoneal injection. Most of the rats used for this purpose were sacrificed 3 hours after injection. No difference was found in the sodium partition whether the period was 1, 3, or 24 hours, as had already been noted by Greenberg, Campbell, and Murayama (9). To determine the radioactivity of the samples, appropriate weights of tissue were dry-ashed in porcelain dishes, and the ash dissolved in hydrochloric acid and transferred to 0.25 ounce ointment capsules, which had been varnished to prevent reaction with the acid, and evaporated to dryness. The radioactivity was measured with a Geiger-Müller counter and the mica-walled counter tube described by Copp and Greenberg (10).

The apparent extracellular phase distribution  $(\text{H}_2\text{O})_E$  of Cl and of  $\text{Na}^{22}$  was calculated by means of the following equations.

$$(\text{H}_2\text{O})_E\text{Cl} = \frac{\text{tissue Cl}}{\text{serum Cl}} \times 100 \quad (1)$$

$$(\text{H}_2\text{O})_E\text{Na}^{22} = \frac{\text{tissue Na}^{22} (\% \text{ of dose per gm.})}{\text{serum Na}^{22} (\% \text{ of dose per ml.})} \times 100 \quad (2)$$

The values of apparent extracellular phase were calculated in terms of both the fresh tissue weight concentrations and in terms of the concentrations per kilo of water. It will be noted that a correction factor (usually 0.95) for the Donnan ratio between blood plasma and interstitial fluid, commonly incorporated in the equations for calculating the extracellular phase (9, 11), has been left out of Equations 1 and 2 for the reason that the magnitude of this factor, uncertain at best, is rendered still more doubtful by the disturbed electrolyte distribution in the chloride-deficient animals. Equations 1 and 2 are sufficient for the determination of the nature of the alteration in the body fluids caused by chloride deprivation.

Calculation also was made of the apparent volume of distribution of the sodium in the body by means of the equation

$$(\text{H}_2\text{O})_E \text{Na}^{22} = \frac{100 \text{ (= administered Na}^{22}\text{)}}{\text{serum Na}^{22} \text{ (\% of dose)}} \times \frac{100}{\text{body weight}} \quad (3)$$

The  $\text{Na}^{22}$  excreted in the 3 hour period of these experiments was insignificant and therefore is omitted from Equation 3.

#### RESULTS AND DISCUSSION

*Growth*—We reported in an earlier paper that chloride-deficient rats failed to grow normally on a diet containing 12 mg. per cent of chloride. On the present diet, containing 2 to 5 mg. per cent of chloride, the failure of growth was more striking. The control male rats reached a weight of about 400 gm. at the age of 18 weeks, at which time the growth curve tended to reach a plateau. The subsequent growth was very slow, although some of the rats eventually attained a weight of 500 gm. or more. On the other hand, the deficient animals very early fell behind the controls, the weight reaching a plateau of about 130 gm. at 12 weeks of age. They may attain a weight of 200 to 300 gm., but only after a period of 8 to 12 months.

This is not entirely due to decrease in appetite, because three pair-fed control rats, restricted to the same food intake as litter mate deficient animals, gained more than twice as much weight over a period of 10 weeks. Each group of three rats consisted of two males and one female. The controls gained 171 gm. per rat and the chloride-low rats only 73 gm. The growth curves are shown in Fig. 1. In other words, the chloride-deficient rats utilize their food inefficiently and gain less weight per gm. of food ingested than do the controls.

*Chloride*—The results of the chloride analysis of blood, tissues, and carcass are given in Table I. As was reported formerly, the concentration of chloride in the blood is considerably reduced. In the present series, the mean serum chloride level in the control group was 352 mg. per 100 ml. (98.2 mm per liter) as against 265 mg. per 100 ml. (74.6 mm per liter) for the deficient animals.

Tissue chloride analysis showed a reduction in the chloride content of skin, muscle, liver, kidney, brain, testis, stomach, lung, and carcass. This reduction is evident whether chloride content is expressed in terms of fresh weight or of water content. The heart, incisor teeth (fresh weight concentration only), and spleen are the only exceptions. The tissues in which the difference between the means of control and chloride-deficient values is 3 times (or more) the standard error of the differences are muscle (concentration in total water), testis, brain, stomach, spleen (fresh weight), lung, and serum. In skin, muscle, liver, and kidney, the difference is between 2 to 3 times the standard error of the difference and for the last three named tissues this is true only for the fresh weight concentrations. However, the fact that all the differences, with the three exceptions mentioned, are in

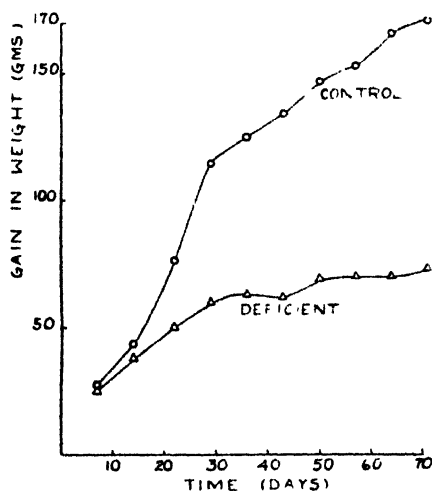


FIG. 1 Growth curves of chloride-deficient and pair-fed control rats. Mean weight gains of two males and one female in each group on the same food intake.

the same direction lends significance to the decreased values for the other tissues.

The results of the calculations of the apparent extracellular phase (chloride space) from the chloride values are given in Table II. In chloride deficiency, the apparent extracellular phase is increased in the skin, muscle, liver, kidney, brain, heart, testis, femur, lung, incisors, stomach, spleen, and carcass (per cent of fresh weight only). With the exception mentioned, this is true whether the chloride space is calculated in terms of fresh tissue weight or of water content.

The heart of the deficient animals shows a particularly large increase of the chloride space, the value being about double that of the control. It is to be noted that this high value results from the fact that the chloride con-

tent of the heart is actually increased in the deficient animals, while the serum chloride is decreased. The difference between the mean values for the control and chloride-deficient animals is 3 times (or more) the standard error of the difference with respect to both units of concentration for muscle, liver, testis, heart, spleen, and lung and on the basis of fresh weight only for skin, kidney, and incisors. The difference is between 2 and 3 times the standard error of the difference for brain, stomach, and carcass (fresh

TABLE I  
*Chloride Content of Tissues in Normal and Chloride-Deficient Rats*

Tissue	Controls*			Chloride-low*			Standard error of difference	
	No. of animals	Cl per 100 gm. fresh tissue	Cl per kilo H <sub>2</sub> O	No. of animals	Cl per 100 gm fresh tissue	Cl per kilo H <sub>2</sub> O		
		mg	mM		mg.	mM	mg. per 100 gm	mM per kg H <sub>2</sub> O
Blood serum†	44	352 ± 2.8		33	265 ± 7.8			
Skin. . . .	30	152.4 ± 6.7	91.0 ± 4.2	34	139 ± 5.7	78.6 ± 4.4	6.8	6.0
Muscle . . .	20	37.8 ± 1.2	15.2 ± 0.4	24	33.8 ± 1.1	11.6 ± 0.5	1.6	0.6
Liver. . . .	20	78.2 ± 3.6	32.4 ± 1.4	30	70.5 ± 3.8	28.2 ± 4.9	5.2	5.0
Kidney. . .	21	172 ± 8.1	66.2 ± 2.6	20	148 ± 5.6	60.3 ± 2.8	9.9	3.8
Brain . . .	5	122 ± 0.95	45.0 ± 0.5	3	91.2 ± 4.8	34.4 ± 1.3	4.9	1.4
Heart . . .	4	158 ± 4.1	57.8 ± 4.8	4	188 ± 16.4	66.2 ± 3.1	17.0	5.7
Testis . . .	5	200 ± 6.4	69.0 ± 3.0	5	168 ± 9.7	57.2 ± 2.7	11.6	4.1
Stomach. . .	3	170 ± 9.8	66.8 ± 4.2	3	129 ± 5.0	49.3 ± 1.6	11.0	4.5
Spleen . . .	3	137 ± 11.0	51.6 ± 5.2	3	147 ± 6.6	61.4 ± 0.7	12.8	5.3
Lung. . . .	3	184 ± 8.2	67.6 ± 3.2	3	141 ± 13.0	51.6 ± 4.2	12.5	5.3
Bone (femur)	4	114.8 ± 8.3	89.4 ± 10.5	7	117 ± 10.4	84.0 ± 8.9	13.3	13.8
Teeth (incisor)	2	135 ± 11.5	57.4 ± 12.7	3	170 ± 42.7	46.3 ± 10.0	43.3	
Carcass. . .	3	100 ± 15.5	67.4 ± 3.7	5	99.6 ± 20.0	49.3 ± 9.8	25.3	10.4

\* The values are means plus or minus the standard error.

† The blood serum values are in mg. per 100 ml.

weight). Again, the differences all being in the same direction, the significance of the lesser values is increased. In the case of the carcass, the greater value of the chloride space as per cent of fresh weight is at least partly a result of the extra fat content of the control rats (2). Analysis of the fat contents of six control and six deficient rats of the earlier group of the present series yielded mean values of  $28 \pm 4.1$  and  $16.6 \pm 4.2$  per cent respectively. The difference of the means, 4.8 times the standard error of the difference, is highly significant.

*Na<sup>22</sup> Distribution*—From the distribution of Na<sup>22</sup>, it was calculated that chloride deficiency increases the apparent extracellular phase (sodium space) in the skin, muscle, liver, kidney, brain, heart, testis, femur, carcass, and body as a whole. The results are given in Table III. All the differences, as with chloride, are in the same direction. The difference between the means of the control and deficient animals is 3 times (or more) the standard error of the difference for muscle, kidney, femur, and carcass

TABLE II

*Apparent Volume of Distribution of Chloride in Per Cent in Tissues of Normal and Chloride-Deficient Rats*

Tissue	Controls			Chloride-low			Standard error of difference	
	No. of rats	Fresh weight	Water content	No. of rats	Fresh weight	Water content	Fresh weight	Water content
Carcass...	3	23.5 ± 4.0*	66.8 ± 1.7	5	36.1 ± 2.2	62.6 ± 3.6	4.6	6.0
Skin	27	45.0 ± 1.9	96.0 ± 3.5	28	55.9 ± 2.4	103 ± 14.1	3.3	5.9
Muscle	18	11.4 ± 0.4	15.3 ± 0.5	17	13.7 ± 0.5	18.0 ± 0.6	0.65	0.8
Liver	17	23.2 ± 2.1	33.3 ± 1.4	17	32.3 ± 1.9	46.1 ± 2.6	2.8	3.0
Kidney	17	52.4 ± 2.0	69.7 ± 2.7	15	62.1 ± 2.6	77.6 ± 4.1	3.3	4.9
Brain	5	36.7 ± 0.7	50.0 ± 2.1	4	50.2 ± 4.8	64.6 ± 6.1	4.9	6.5
Heart	5	35.2 ± 4.6	41.0 ± 5.8	6	66.9 ± 5.1	87.8 ± 8.1	6.9	10.0
Testis	3	60.7 ± 2.4	72.6 ± 5.2	3	82.6 ± 2.8	102 ± 3.1	3.7	6.1
Stomach	3	50.4 ± 2.6	71.6 ± 3.7	3	68.7 ± 7.4	92.7 ± 9.0	8.0	9.7
Spleen...	3	41.4 ± 3.5	54.0 ± 5.7	3	77.0 ± 3.7	110.0 ± 10.3	5.1	11.8
Lung	2	55.2 ± 2.2	72.0 ± 3.0	3	73.1 ± 0.5	96.5 ± 1.7	2.2	3.5
Bone (femur)	1	39.4	126	3	71.5 ± 5.4	205 ± 18.5		
Teeth (incisor)	2	40.0 ± 3.5	609 ± 190	3	84.9 ± 15.2	802 ± 216	5.9	287

\* The measure of variability is the standard error of the mean.

(the last two in per cent of wet weight only), and 2 to 3 times for liver, testis, and carcass (in per cent of water content).

Comparison of the values for the apparent extracellular phase obtained from the chloride analyses with those from the partition of radioactive sodium yields deductions of considerable interest. The values for the chloride space, with the exception of that for muscle, femur, and carcass, are higher than the sodium space values for the corresponding tissue. The muscle values are identical within the range of the error of the methods, the values for the carcass are practically the same, and the differences in the case of the femur are small (Tables II and III).

As the calculated extracellular phase of muscle by both methods is the same, while for other tissues the chloride space is consistently higher, it seems reasonable to surmise that in these tissues some of the chloride is either in an intracellular phase (or at least outside the sodium space) or else it is present in some form in the extracellular fluid which is not in mobile equilibrium with the blood plasma.

Some support for this interpretation is offered by the experiments of Amberson *et al.* (12) on the chloride exchange following perfusion of cats with a chloride-free fluid ( $\text{SO}_4^{--}$  substituted for  $\text{Cl}^-$ ). A rapid equilibrium with the chloride of the blood plasma occurred in red corpuscles, muscle,

TABLE III  
*Apparent Volume of Distribution of  $\text{Na}^{22}$  in Per cent in Tissues of Normal and Chloride-Deficient Rats*

Tissue	Controls			Chloride-low			Standard error of difference	
	No. of rats	Fresh weight	Water content	No. of rats	Fresh weight	Water content	Fresh weight	Water content
Whole body	22	$26.7 \pm 0.7^*$		24	$33.7 \pm 1.2$		1.4	
Carcass ..	12	$25.4 \pm 1.0$	$42.8 \pm 1.4$	10	$30.9 \pm 1.1$	$47.9 \pm 1.8$	1.5	2.3
Skin. ....	19	$14.9 \pm 1.2$	$31.5 \pm 2.4$	20	$16.5 \pm 1.7$	$32.4 \pm 3.3$	7.3	4.1
Muscle ..	19	$10.3 \pm 0.5$	$13.9 \pm 0.7$	19	$14.7 \pm 1.0$	$20.2 \pm 1.3$	1.1	1.5
Liver..	17	$13.8 \pm 0.8$	$20.0 \pm 1.1$	20	$16.9 \pm 0.9$	$22.9 \pm 1.2$	1.2	1.6
Kidney ..	18	$32.6 \pm 1.8$	$42.7 \pm 2.5$	19	$44.8 \pm 2.2$	$57.7 \pm 2.4$	2.9	3.5
Brain ..	7	$21.5 \pm 3.9$	$27.6 \pm 4.5$	9	$24.4 \pm 1.7$	$31.6 \pm 2.1$	4.3	5.0
Heart ..	3	$29.1 \pm 3.9$	$36.8 \pm 5.5$	4	$32.4 \pm 3.0$	$41.2 \pm 3.9$	4.9	6.7
Testis	4	$18.1 \pm 2.1$	$18.9 \pm 2.5$	4	$36.6 \pm 7.5$	$48.3 \pm 12.9$	7.8	13.2
Bone (femur)	18	$43.7 \pm 3.5$	$141.2 \pm 12.0$	20	$57.8 \pm 2.6$	$144.9 \pm 9.4$	4.4	15.4

\* The measure of variability is the standard error of the mean.

lung, kidney, spleen, heart, and liver. Most of their chloride is retained even at low plasma levels by the cerebrum, cerebellum, and spinal cord; tendon, skin, and bone retain much and the stomach retains about one-third of the chloride content. When tissue chloride was plotted against plasma chloride concentrations, a series of straight lines at different levels but having the same slope was obtained for stomach, spleen, salivary gland, pancreas, small intestine, heart, and liver. The identity of slope was considered to indicate that the diffusible chloride has the same concentration in all of the above tissues and that the difference in levels is a measure of the difficultly diffusible chloride content (in the order given with the stomach at the top and the line for the liver passing through the origin).

The observations of Hiatt (13) on hypochloremia induced by nitrate diuresis are not in good accord with those mentioned above. He found that the chloride in dog tissues (cerebrum, bone, muscle, lung, ventricle, liver, spleen, pancreas, adrenal, kidney, tendon, stomach, and duodenum) varied directly with the plasma chloride. Some suggestions to explain the different results are as follows: As Amberson *et al.* (12) have suggested, nitrate may exchange with intracellular chloride or the much longer time of Hiatt's experiments allows the sluggishly diffusible chloride to approach equilibrium. Determination of the rate of reentry of chloride (after intravenous administration of NaCl) showed a delay in the brain and spinal fluid, indicating that this ion encounters resistance in entering the nervous tissue (13).

Experiments with radioactive sodium have shown that its penetration into the sodium phase of skin, kidney, liver, and muscle is rapid but is delayed in the testes, femur, and brain (9, 11). Manery and Bale do not feel that the sodium in the rapidly penetrated tissues is necessarily all extracellular nor that delayed penetration is an infallible indication of an intracellular distribution. In the kidney, in which penetration is rapid, they point out that some of the sodium must enter the cells in the process of excretion and of reabsorption through the tubules. Delayed penetration may possibly be only an indication of a barrier (as in the case of the brain) interposed between the blood and the extracellular phase.

The calculated values of the extracellular phase indicate that the organs of the chloride-deficient rats have a slight edema. The increase in extracellular fluid volume is not merely apparent, owing to the retention of chloride, but is supported by the increases observed with  $\text{Na}^{22}$ . This fits in with the conclusion of Voris and Thacker that the cells of chloride-deficient rats are water-poor, because any increase in the extracellular fluid volume, calculated as per cent of the total water content, involves a decrease of the intracellular fluid volume. The increase appears to be an absolute one as well as a relative increase at the expense of the intracellular fluid. This is indicated by the accompanying increase which is obtained when the extracellular phase is calculated as per cent of fresh weight of tissues.

*Alkalosis*—A number of experiments were carried out to obtain more information on the changes in the blood and on the state of alkalosis which accompany the chloride deficiency. Because of the previously made observation that the increase in total carbon dioxide does not balance the decrease of the chloride of the blood (1), the total base content of the serum was determined on twelve control and twelve deficient rats by the electro-dialysis method of Joseph and Stadie (14). These determinations revealed that there is no decrease in the total base of the blood to balance the loss



of chloride. This implies that there is an increase in some (still unknown) anion of the blood.

It was expected that if an alkalosis is present in the chloride-deficient animals, even though compensated, there would be an increase in the citrate excretion, and this was found to be the case. The results are recorded in Table IV. The control males excreted an average of 0.44 mg. of citrate per rat per day in contrast to the mean of 7.5 mg. for the deficient animals. These values include a variable amount of citrate from the seminal fluid which is high in citrate.

*Histopathology*—During autopsies it was noticed that the kidneys were lighter in color in the deficient rats and were covered with translucent spots. Their appearance suggested fatty degeneration but this was found not to be the case when frozen sections (fixed in formalin) were made and stained with scarlet red and iron hematoxylin. Sections fixed in Bouin's solution embedded in celloidin and stained with hematoxylin and eosin or iron hematoxylin-aniline blue showed considerable kidney damage. This consisted of degeneration of the glomeruli to cyst-like structures with flat

TABLE IV  
*Urinary Excretion of Citric Acid in Mg. per 24 Hours per Rat*

Controls			Chloride-low			Standard error of difference
No. of rats	Range	Mean $\pm$ S. E.	No. of rats	Range	Mean $\pm$ S. E.	
13	0-0.9	0.44 $\pm$ 0.07	42	0.3-24.4	7.5 $\pm$ 0.9	0.9

epithelium containing varying amounts of compact cellular material. The nuclei in these glomeruli were deteriorated and in some there was complete replacement by scar connective tissue. The epithelial cells of the convoluted tubules were for the most part swollen and the nuclei small, irregular, compact, and sometimes pycnotic. In the medulla many collecting tubules were degenerating, as is shown by the dissolution of the epithelial cells, gaps in the tubular walls, and lumen filled with homogeneous masses. In both cortex and medulla there was increased interstitial tissue, so that the remaining tubules were considerably separated. In many places tubules were replaced with scar tissue. A more detailed description of the pathology will be published elsewhere.

Most of these animals had been on the experimental diet 8 months or more but kidneys have been found with a high degree of degeneration after only 1 month on the deficient diet.

The specific cause of the kidney damage, of course, is difficult to ascertain. The diet of the deficient rats differs from that of the controls only

in the salt mixture and there only in the chloride and carbonate concentrations. The high bicarbonate concentration alone cannot be blamed, because Addis, MacKay, and MacKay (15) kept rats on a high (4 per cent) sodium bicarbonate diet for 300 days with no resulting kidney pathology. It may be possible that the extreme conservation of chloride (1) overworks the kidney. Or perhaps the adrenal is influenced by the altered salt metabolism and thus the kidney is affected indirectly.

We are indebted to Professor E. O. Lawrence and the staff of the Radiation Laboratory of the University of California for kindly supplying us with  $\text{Na}^{22}$  and to Miss Virginia Pallais and Mr. Charles Davenport for their help in the preparation and analysis of the histological sections.

#### SUMMARY

1. Rats were raised from the age of 3 to 4 weeks on a diet containing 2 to 5 mg. per cent of chloride and 2 per cent carbonate and bicarbonate to replace the chloride. These rats showed a considerable reduction in growth. Control rats, pair-fed with litter mate deficient animals, gained more than twice as much weight over a period of 10 weeks.

2. The blood serum chloride values were reduced from 98.2 mm per liter in the controls to 74.6 mm per liter in the deficient rats. Tissue chloride content was reduced in the skin, muscle, liver, kidney, testis, brain, stomach, lung, and carcass. There were increases in the heart and spleen. The chloride space was increased in skin, muscle, femur, liver, kidney, testis, brain, heart, carcass, stomach, spleen, lung, and incisors. Sodium space was increased in skin, muscle, femur, liver, kidney, testis, brain, heart, carcass, and the body as a whole.

3. Urinary citrate excretion increased from a mean of 0.44 mg. per rat per day to 7.5 mg.

4. There is extensive kidney damage which in the late stages involves the whole nephron. There is degeneration and replacement by scar tissue. In spite of these changes some of the deficient rats lived a year or longer.

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# A BIOASSAY FOR LYSINE BY USE OF A MUTANT OF *NEUROSPORA*

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The amount of lysine present in protein hydrolysates has been determined by several methods. The classical one is the procedure introduced by Kossel and Kutscher (1). In this method arginine and histidine are removed as silver salts, and lysine is precipitated as its phosphotungstate and isolated as the picrate. This scheme has been variously modified by several investigators (2-4). Other methods which have been used are the nitrogen distribution method introduced by Van Slyke (5) and the electrical transport procedure proposed by Foster and Schmidt and further developed by Albanese (6). In view of the fact that these procedures are largely limited to analyses involving pure protein hydrolysates, and in view of the criticisms of these methods expressed by various workers (7-10), it seems clear that a micromethod suitable for use in connection with protein hydrolysates and more complex mixtures would be of value.

In the last 2 years a considerable number of publications have shown the usefulness of microorganisms for the purpose of estimating amino acids (11-19), and very recently several microbiological procedures for determining lysine have been proposed (20-22). The present paper describes the use of a mutant of *Neurospora crassa* in an assay for this amino acid.

*Neurospora* offers a decided advantage over the usual test organisms in the simplicity of its nutritional requirements, for in addition to inorganic salts and a carbon source it requires only biotin. Mutant strains have been produced, however, which require a single additional growth substance (23). It has been shown that one of these mutants, strain 4545, requires lysine in addition to biotin and that its growth is specifically a function of the available lysine (24). The simplicity of this requirement has made it possible to analyze the difficulties involved in the bioassay for lysine.

The basal medium used for these experiments is given in Table I. Although this mutant fails to grow in the absence of lysine, it has been found that the addition of other amino acids in the presence of lysine will stimulate heavier growth (24). This is interpreted as a sparing action exercised on the lysine. Extensive experiments have shown that asparagine and glutamic acid, when added as indicated in Table I, will stimulate to the maximum, thus making the amount of lysine the only limiting factor.

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Alkali-hydrolyzed zein was also used to supplement the medium in order to test whether a more diverse supplement might alter the assay values. In these experiments a supplement of hydrolyzed zein at a level of 2.5 mg. per ml. and asparagine at a concentration of 0.5 mg. per ml. was used instead of the supplement given in Table I. Three protein hydrolysates were assayed simultaneously on the two media. The average difference

TABLE I  
*Composition of Basal Medium*

This medium may be made up in 5 gallon Pyrex carboys, fitted with rubber stoppers and sterile siphoning apparatus, autoclaved,\* and thus kept over a long period of time.

Ammonium tartrate, gm. . . . .	5.0
"    nitrate, " . . . . .	1.0
Monobasic potassium phosphate, gm. . . . .	1.0
Magnesium sulfate (7H <sub>2</sub> O), gm. . . . .	0.5
Sodium chloride, gm. . . . .	0.1
Calcium " (anhydrous), gm. . . . .	0.1
Sucrose, gm. . . . .	20.0
Biotin, " . . . . .	$5.0 \times 10^{-6}$
Boron,† mg. . . . .	0.01
Molybdenum,† mg. . . . .	0.02
Iron,† mg. . . . .	0.20
Copper,† mg. . . . .	0.10
Manganese,† mg. . . . .	0.02
Zinc,† mg. . . . .	2.00
Phosphate buffer, 0.5 M, pH 5.5, ml. . . . .	100
Asparagine,‡ gm. . . . .	1.0
Glutamic acid‡ . . . . .	1.0
l(+)-Lysine (calculated as free base), mg. . . . .	5.84
Water, to bring volume to 1.0 liter	

\* Slight caramelization may occur, but this makes no noticeable difference in the results.

† As salts.

‡ Adjusted in solution to pH 7.0 before addition to the medium.

between the lysine values was less than 2 per cent, and the largest difference was 4 per cent.

Stocks of strain 4545 have been kept on slants containing the basal medium, plus agar 2 per cent, casein hydrolysate 50 mg. per cent, yeast extract 0.5 per cent, malt extract 0.5 per cent, and l(+)-lysine 10 mg. per cent. The cultures are incubated on this medium for 5 to 6 days. At the end of this time enough conidia should be present for use in inoculating a series of cultures.

Cultures for assay are made in 125 ml. Erlenmeyer flasks, with 25 ml.

of basal medium per flask. Varying amounts of *l*(+)-lysine ranging from 0 to 0.6 mg. (calculated as the free base) are added to make up a standard curve. For dependable and accurate assays the range 0.1 to 0.5 mg. of lysine per flask should be used. When the concentration of lysine is above 0.6 mg. per 25 ml., the variability increases markedly, and when determinations are made below 0.1 mg., chance errors affect individual determinations too much. The natural isomer of the amino acid should be used throughout, since *dl*-lysine is approximately, but not exactly, one-half as active as *l*(+)-lysine (24). Fig. 1 shows a typical standard curve.

Material to be assayed must be hydrolyzed, and this may be accomplished by any standard method which does not racemize the amino acids.

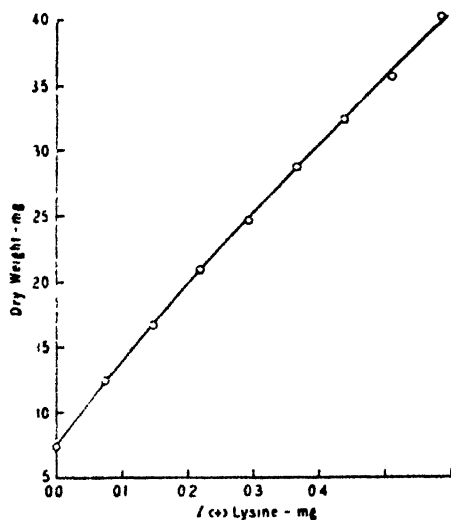


FIG. 1. A standard curve for the assay of lysine with *Neurospora* mutant, strain 4545. The points are averages of duplicate determinations.

If sulfuric acid is used, it should be removed with barium hydroxide. The precipitate of barium sulfate must be thoroughly washed to prevent losses by adsorption. If hydrochloric acid is used, it should be largely removed by vacuum distillation before the pH is adjusted to 5.5 to 6.5 with potassium hydroxide. It is desirable that the final concentration of material be 20 mg. per ml. or higher. No special precautions need be taken to remove the humin.

In a previous description of strain 4545 (24), it was shown that arginine is a specific inhibitor for the *lysineless* mutants. For this reason it is necessary to remove the arginine from the material being assayed or to hydrolyze it to ornithine, for ornithine is inhibitory only if present in much higher concentration. Arginine, if present in equimolar amount with

lysine, will, at a pH of 5.5, reduce the dry weight of a 96 hour culture to one-half that of the control with no arginine. In comparison, the concentration of ornithine must be 28 times as high to produce the same effect.

Two methods were tried in order to accomplish the removal of arginine. The arginine was precipitated as the silver salt in the manner described by Kossel and Kutscher (1). This method gave fairly reliable results, but the procedure is not simple, and occasionally inconsistent results were obtained. In the author's experience, a more convenient and reliable method is that of hydrolyzing the arginine with arginase. The latter method has been used throughout the experiments reported here.

A preparation of an arginase concentrate from liver has been made by a process similar to that of Hunter and Dauphinee (25). A quantity of liver<sup>1</sup> purchased in the market is ground and shaken vigorously with an equal weight of water for 10 minutes. The container is then placed in a water bath at 62–65°, and constantly stirred. The mixture is allowed to come to 58° and is maintained at that temperature for 5 minutes, whereupon it is cooled under the tap. The suspension is placed in a fluted filter paper and allowed to filter overnight. The filtrate is adjusted to pH 7.0. It may then be evaporated to dryness by exposing it on a large clean glass surface. The dried extract is scraped off, dried further in a desiccator, and ground to a powder. A preparation of this nature was used in this laboratory over a period of 3 months without noticeable loss in arginase activity.

An experiment to test whether the arginase preparation causes any destruction of lysine or whether it contains important amounts of lysine is shown in Table II. Only 4  $\gamma$  of lysine were found per mg. of the preparation: this amount is not sufficient to alter assay results significantly. Neither does any measurable loss of lysine activity result from incubation with the preparation.

For the destruction of arginine, 0.5 gm. of hydrolyzed material is placed in a 50 ml. volumetric flask and 25 mg. of arginase preparation are added, together with 20 ml. of 0.25 M pyrophosphate buffer. The pH of the solution is adjusted to 8.5 to 9.0 with normal sodium hydroxide and the total volume brought to approximately 48 ml. The mixture is covered with toluene, incubated at 35° overnight, and, in the morning, adjusted to pH 5.5 to 6.5 with 12 N sulfuric acid. Finally, the volume is brought to 50 ml. with water, and the solution is placed in a boiling water bath for 15 minutes and filtered. The filtrate, containing 10 mg. per ml., is used directly in the assay. The filtrate may be preserved under toluene for future reference.

In performing an assay experiment, a standard curve is set up as de-

<sup>1</sup> Hog liver was used in the experiments described here.

scribed previously. It is advisable to run a standard series with each assay, since a certain amount of variability in the standard curve is to be expected. The hydrolysate is distributed among 125 ml. Erlenmeyer flasks and 25 ml. of the basal medium are added to each. A range of concentrations wide enough to insure having at least two levels in the assay range should be used. The flasks are plugged with cotton, autoclaved at 15 pounds steam pressure for 10 to 15 minutes, and, after being cooled, are inoculated with 1 or 2 drops of conidial suspension of the *lysineless* mutant. The cultures are incubated for 6.5 to 7.5 days. The mycelium is then removed with a needle or forceps, pressed as dry as possible on filter paper, dried for 3 hours in an oven at 75–90°, and weighed. The

TABLE II

*Effect of Arginase Preparation on Lysine*

To each of three 10 ml. volumetric flasks (A, B, C) were added 4 ml. of 0.25 M pyrophosphate buffer (pH 9.0) and the substances indicated. All were adjusted to 10 ml. with water, covered with toluene, and incubated for 18 hours at 35°. They were finally assayed for lysine with the *Neurospora* mutant.

Flask	Lysine added	Arginase preparation	Aliquot assayed*	Lysine, found	Lysine, theoretical
	mg	mg	ml	mg	mg.
A	7.3	0	0.2	0.160	0.146
			0.5	0.365	0.365
B	0.0	50	2.0	0.036	
C	7.3	50	0.2	0.153	0.150†
			0.5	0.379	0.374†

\* Aliquots assayed in triplicate.

† Theoretical lysine is equal to the lysine added plus the lysine in the arginase preparation.

weights from the standard series are plotted and the assay values are calculated from the standard curve in the usual manner.

The lysine contents of five hydrolysates were determined by this method. The results are shown in Table III together with values cited in the literature for comparison.

The reproducibility of values obtained by this method is shown by the experiments described in Table IV. Lysine values determined on the same material at different times agree with each other within 4 per cent.

Table V shows the result of recovery experiments. The recovery of added lysine is between 97 and 109 per cent. It is noteworthy that the recovery of lysine added to hydrolyzed *Neurospora* mycelium was 101 per cent, indicating that the method is applicable to determinations on very complex mixtures.



TABLE III  
*Lysine Content of Various Hydrolysates*

Material	Nitrogen in sample*	Ash content	Lysine†	Values from literature‡	Remarks
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Casein Hydrolysate 1	15.40	3.92	6.6	6.39 (9) 8.3 (22) 6.0 (26) 7.5 (27)‡	Smaco, vitamin-free preparation
“ “ 2	15.40	3.92	6.8		“ “
Ovalbumin	14.83	4.58	6.3	4.96 (9) 6.4 (26) 5.0 (27)‡	Merck, technical
Gelatin	18.14	1.79	6.9	5.92 (9) 5.9 (26) 4.5 (27)‡	Difco, Bacto-gelatin
Gluten	14.35	2.33	1.3	1.9 (27)‡	Pfanstiehl

\* Corrected for ash and moisture.

† The numbers in parentheses refer to bibliographical references.

‡ The values refer to those considered "best values" by Block and Bolling

TABLE IV  
*Comparison of Results of Separate Assays of Same Hydrolysates*

Aliquots of ovalbumin and casein hydrolysates were treated with arginase preparation on three separate occasions. The three samples were assayed at five different times

Material	Arginase Treatment No	Assay Experiment No	Lysine*
			<i>per cent</i>
Ovalbumin	1	1	6.38
	1	2	6.31
	2	3	6.30
	2	4	6.40
	3	5	6.32
Average .....			6.34
Casein Hydrolysate 2	1	1	6.79
	1	2	6.66
	2	3	6.92
	2	4	6.73
	3	5	6.87
Average .....			6.79

\* Corrected for ash and moisture.

If lysine is the only factor limiting the final amount of growth, lysine assays at various levels of a protein hydrolysate should not vary beyond the experimental errors. To test this a standard series was set up simul-

TABLE V  
*Recovery of Lysine Added to Hydrolyzed Materials*

Material	Sample*	Lysine in sample†	Lysine added	Total lysine found	Lysine recovered	Recovery
		(a)	(b)	(c)	(c-a)	
	mg.	mg.	mg.	mg.	mg.	per cent
Casein Hydrolysate 1	2.74	0.179	0.292	0.490	0.311	106
“ “ 2	2.74	0.186	0.292	0.490	0.304	104
Ovalbumin	2.56	0.164	0.292	0.473	0.309	106
Gluten	9.04	0.120	0.292	0.404	0.284	97
Gelatin	2.61	0.180	0.292	0.499	0.319	109
<i>Neurospora</i> mycelium	10	0.236	0.292	0.531	0.295	101

\* Corrected for ash and moisture, except in the case of *Neurospora* mycelium.

† Calculated from *Neurospora* assay carried out simultaneously.

TABLE VI  
*Lysine Determinations at Various Levels of Gluten Hydrolysate*

Hydrolyzed gluten	Lysine found	Lysine*
mg.	mg.	per cent
5	0.064†	1.42†
5	0.075†	1.66†
10	0.123	1.36
10	0.120	1.33
15	0.180	1.33
15	0.180	1.33
20	0.242	1.34
20	0.240	1.33
25	0.302	1.34
25	0.313	1.38
30	0.375	1.38
30	0.356	1.32
Average.		1.34

\* Corrected for ash and moisture.

† Not included in the assay average, since they did not fall between 0.1 and 0.5 mg. of lysine on the standard curve.

taneously with a series having six concentrations of an arginase-treated gluten hydrolysate, and the lysine was determined at each level. The results are shown in Table VI. It is concluded that, over the range covered, lysine is strictly limiting.

A disadvantage inherent in the method as described is the length of the incubation period. The first experiments with this mutant were done with 72 hour cultures. It was found, however, that various factors such as the concentration of certain amino acids would change the rate of growth, and therefore the results from 72 hour cultures. The amount of growth obtained after complete utilization of lysine was unaffected. It was found that under the prescribed conditions the lysine of the medium is completely utilized in 7 days. 7 day cultures were, therefore, used in later experiments.

In an attempt to speed up the assay, experiments were carried out with the Petri plate method used by Thompson, Isbell, and Mitchell (28) in a *Neurospora* assay for *p*-aminobenzoic acid. The experiments showed that, although the sensitivity can in this way be increased 10-fold and the period of assay reduced to 1 day, there are a number of extraneous factors which affect the rate of growth in Petri plates but do not alter the dry weights obtained in flasks. Owing to these complicating factors, the Petri plate technique appeared to be inferior to liquid culture for lysine assay.

The usual procedure in establishing a method for the quantitative determination of an amino acid is to compare the results obtained with the method in question with those obtained by other methods. In the case of lysine, its content in casein has been most commonly used as a basis for comparison. In view of the fact that even the most recently cited figures vary over a range of 6.39 per cent<sup>2</sup> (9) to 8.3 per cent (22), it seems clear that a critical comparison of methods can be made only after the results have been obtained from a common hydrolysate.

Nevertheless it is possible to show that an assay method gives reliable results by less direct evidence. In this regard the *Neurospora* method appears to have satisfactory support. The reproducibility of results in separate experiments is shown to be good. The recovery experiments are within the limits accepted for microbiological procedures. And assays at different points on the standard curve give the same lysine values. Therefore, the method described would seem to be applicable to the determination of lysine.

#### SUMMARY

A microbiological method for the determination of lysine by use of a mutant of *Neurospora crassa* is described. The inhibition of growth which characterizes *lysineless* mutants of *Neurospora* is overcome by the hydrolysis of arginine to ornithine by a preparation of liver arginase. It is shown that the results are reproducible, that they do not vary significantly over the assay range of the standard curve, and that good recoveries of added

<sup>2</sup> Calculated from Table IV, p. 354, Cohn and Edsall (9).

lysine are found. Lysine values of four protein hydrolysates as determined by the *Neurospora* method are given.

The author wishes to express his appreciation to the members of the genetics laboratory of Stanford University for their constant help in the course of these experiments.

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# ACTIVITY MECHANISM OF YEAST EXTRACTS IN STIMULATING RESPIRATION

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Cook *et al.* (1) showed that alcoholic extracts of yeast cells increased the oxygen uptake of skin, liver, and yeast, it being possible to secure by chemical means partial separation of these activities into three fractions responsible for the results (1, 2). It has also been shown that similar materials exist in extracts of other cells including liver, kidney, spleen (3), and some plant cells (4). Yeast cells, when injured with ultraviolet radiations, produced additional quantities of these substances, an optimum exposure existing for maximum stimulation (5).

Recently, in an attempt to study the mechanism of the action of these principles, the yeast extract was found to stimulate the action of the iron-containing enzyme peroxidase in the oxidation of pyrogallol to purpurogallin (6) and also the enzyme catalase in the splitting of  $H_2O_2$  (7, 8). This stimulation was shown to be a direct excitation of the enzyme, since the extracts possessed no catalase and only a slight peroxidase activity. It was suggested that the yeast extracts contained metal complexes which function by activating the enzyme. This seems to be supported by experiments on such complexes.

It was decided to investigate the respiratory stimulation with the idea that the mode of action might be similar to peroxidase and catalase excitation. It is known that respiratory inhibitors such as KCN and  $NaN_3$  selectively attack the cytochrome oxidase, while narcotics and anesthetics inhibit the cytochrome-reducing system which includes the dehydrogenases, the pyridino-protein enzymes, and the flavoproteins. With the use of these inhibitors it was thought possible to locate the point of action of the yeast extract in the respiratory chain.

## EXPERIMENTAL

The aqueous alcoholic extract corresponds to Sample A prepared as described previously (1). Experiments were carried out with bakers' yeast (*Saccharomyces cerevisiae*) in Warburg respirometers. Respiration measurements were made at 30° in Ringer-phosphate-glucose solution (0.02 per cent glucose) at pH 7.3. The control flasks contained 1 ml. of yeast suspension (4 mg. of dry weight of yeast) and 2 ml. of Ringer-phosphate-glucose solution, and 0.2 ml. of N KOH was placed in the inner

well to absorb  $\text{CO}_2$ . Experimental flasks contained 1 ml. of yeast extract and 1 ml. of the inhibitor diluted in Ringer-phosphate-glucose solution to the desired concentration.

Each inhibitor was tested for antagonism with the extract in two ways. In the first, the solution of the inhibitor was placed in the chamber with the respiring cells immediately before the 15 minute equilibrium period, while the yeast extract (1 ml. containing the desired concentration) was placed in the side arm of the flask and spilled some time after the equilibrium. In the second, 1 ml. of inhibitor was spilled from the side arm, while the yeast extract was present in the chamber.

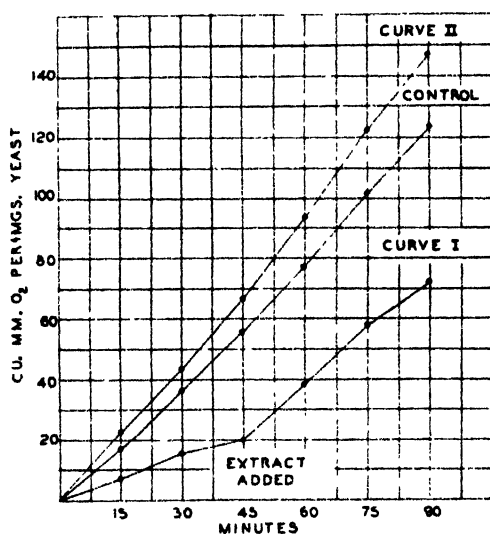


FIG. 1 Effect of yeast extract and potassium cyanide on yeast respiration. Control, yeast + Ringer phosphate-glucose solution; Curve I, same as control + cyanide, extract added at 45 minutes; Curve II, same as control + extract.

In Fig. 1 is shown the antagonistic action of KCN and yeast extract on yeast respiration. Difficulty was encountered with KCN distilling from the side arm or chamber into the KOH well, but this was eliminated as suggested by Krebs (9) by adding a small amount of KCN in the KOH well (1 ml. of 0.1 M KCN + 5 ml. of N KOH). When KCN (M/24,000) was present alone with the respiring cells (Curve I), the respiration was depressed from that of the control. When the yeast extract (6 mg. per ml.) was spilled at the 45 minute period, the rate of respiration increased almost to that of the control, as shown by an immediate rise in the curve. In Curve II, yeast extract was present in the chamber alone with the respiring cells throughout the entire experiment. In some experiments, KCN

was spilled from the side arm while the cells were respiring in the presence of yeast extract. No decrease in the rate of respiration was obtained.

The antagonistic action with KCN concentrations ranging from  $M/3000$  to  $M/60,000$  was tested. As would be expected, higher concentrations were antagonized to a smaller extent than the lower ones with the same level of yeast extract (6 mg. per ml.).

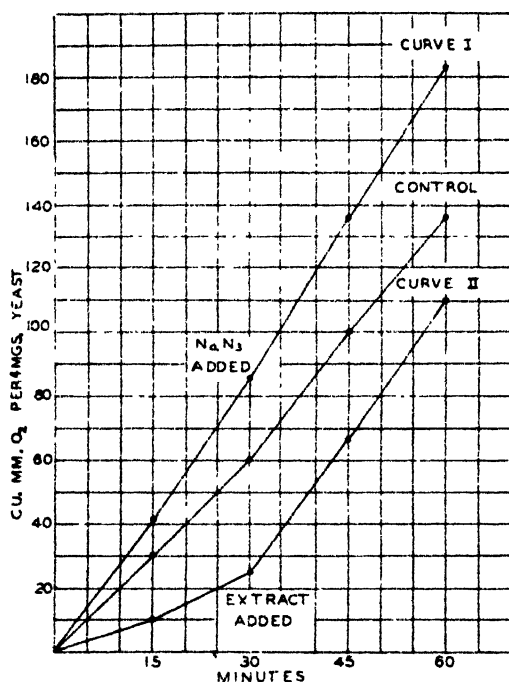


FIG. 2 Effect of yeast extract and sodium azide on yeast respiration. Control, yeast + Ringer phosphate-glucose solution; Curve I, same as control + extract, azide added at 30 minutes; Curve II, same as control + azide, extract added at 30 minutes.

In Fig. 2 is shown the effect of  $\text{NaN}_3$  on yeast respiration when yeast extract is present (6 mg. per ml.). Curve I shows the stimulation of the yeast extract for 30 minutes. At that point 1 ml. of  $\text{NaN}_3$  to make a final concentration of  $M/30,000$  was spilled from the side arm but no change in the rate of respiration was obtained. The  $\text{NaN}_3$  had no effect. Curve II shows the effect of  $M/30,000$   $\text{NaN}_3$  alone on yeast respiration for 30 minutes. 1 ml. of the yeast extract was then spilled and immediately there was a rise in the curve, the rate of respiration increasing to parallel that of the control. The  $\text{NaN}_3$  therefore had no effect when yeast extract was present.



In order to answer the question as to the importance of the dehydrogenases, coenzymes, and flavoproteins in the respiratory chain as affected

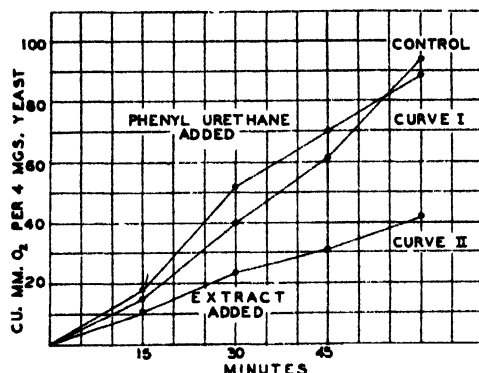


FIG. 3. Effect of yeast extract and phenylurethane on yeast respiration. Control, yeast + Ringer-phosphate-glucose solution; Curve I, same as control + extract, urethane added at 30 minutes; Curve II, same as control + urethane, extract added at 30 minutes.

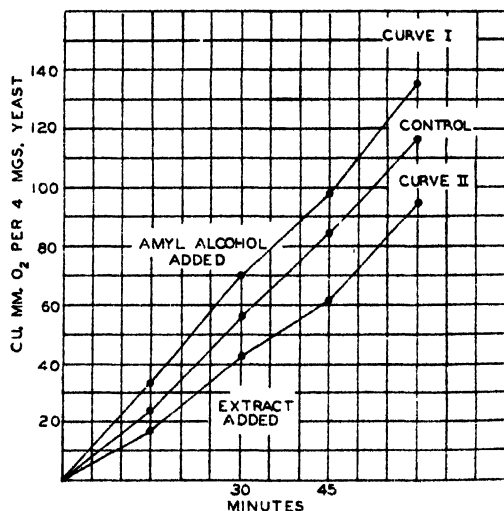


FIG. 4. Effect of yeast extract and amyl alcohol on yeast respiration. Control, yeast + Ringer-phosphate-glucose solution; Curve I, same as control + extract, alcohol added at 30 minutes; Curve II, same as control + alcohol, extract added at 30 minutes.

by yeast extract, it was thought advisable to test the possible antagonism of the extract against such inhibitors as phenylurethane and amyl alcohol which are considered to block the dehydrogenating system.

The inability of the extract (6 mg. per ml.) to antagonize *m*/18 phenylurethane is shown in Fig. 3. When the yeast extract was spilled from the side arm at the 30 minute period (Curve II), no change in the rate of oxygen uptake was obtained. Also, when phenylurethane was spilled from the side arm (Curve I) there was an immediate depression of respiration even though the cells were respiring in the presence of extract. Many experiments were performed with increasing dilutions of the urethane until the amount of depression was extremely slight; yet the yeast extract could not offset the depression. Ethylurethane gave the same qualitative results. With *m*/350 amyl alcohol, Fig. 4, the same inability to offset depression was observed (Curve II). Amyl alcohol, however, was a very unsatisfactory inhibitor for these experiments because of its inability to depress respiration in some cases. This probably explains the inability of amyl alcohol to depress in the presence of the yeast extract (Curve I). However, in no case was the yeast extract able to increase the respiration when the yeast was respiring in the presence of amyl alcohol.

These tests were carried over to rat skin and liver with similar results; *i.e.*, the yeast extract completely offset the depression caused by KCN and NaN<sub>3</sub> on skin and liver as it did for yeast. In the case of skin, the extract had no activity in the presence of urethane or amyl alcohol; however, with liver there seems to be a slight activity. We do not feel that this activity is significant, since stimulation of the liver respiration was obtained with urethane or amyl alcohol itself. Concentrations from *m*/350 to *m*/5600 were tested for both urethane and amyl alcohol with the same qualitative results.

#### DISCUSSION

From the data presented it seems quite clear that the active principles of the yeast extract are definitely effective against KCN and NaN<sub>3</sub> for yeast, skin, and liver, but have no action against urethane or amyl alcohol except possibly a slight one in the case of liver.

It is possible that the antagonism to cyanide and azide and the stimulation of respiration may be caused by different substances in the extract; *i.e.*, certain substances may inactivate the inhibitors and others may accelerate respiration. While this seems likely in the case of certain non-specific inhibitors, such as phenylmercuric nitrate (10), to be discussed in detail in a later paper, we feel that the respiratory effects and the antagonism to cyanide and azide are closely bound together. If this is true, the site of action is indicated as the cytochrome oxidase portion of the chain. This would seem to be supported by the effects of the extract on the similar enzymes, peroxidase (6) and catalase (7, 8), and by the facts that the extract is without effect on succinic dehydrogenase and increases the oxygen uptake

of the cytochrome-cytochrome oxidase-ascorbic acid system of Schneider and Potter (11) (unpublished data). Indeed, the oxidase is by-passed and ascorbic acid is oxidized directly by the extract. This effect and the overcoming of cyanide- and azide-inhibited respiration may perhaps be best explained by the presence in the extract of autoxidizable substances which carry oxygen directly to hydrogen acceptors. This could be fulfilled by such prosthetic groups as the alloxazine nucleotides or pyridine nucleotides, although no positive evidence is available that they are involved in the extract action. These possibilities are under investigation.

#### SUMMARY

Evidence is presented to show that the site of action of yeast extracts active in increasing the respiration of various cells and tissues is the cytochrome portion of the chain, since iron inhibitors could be antagonized by the extract. Inhibitors which attack the dehydrogenating end of the respiratory system could not be antagonized. It is suggested that yeast extracts contain substances which by-pass the cytochrome oxidase or entire cytochrome chain.

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## ENZYMATIC OXIDATION OF URIC ACID\*

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The conversion of uric acid into allantoin involves hydration, oxidation, and decarboxylation. Despite the complexity of this reaction, the enzymatic oxidation of uric acid has generally been assumed to proceed as a single reaction. In contrast to this, permanganate oxidation of uric acid in alkaline solution has long been recognized to involve a number of separate steps. Biltz (1) in 1921, summarizing his own investigations and those of previous workers, concluded that the first step of the reaction is an oxidation at the double bond, leading to an extremely unstable compound which immediately decomposes with the formation of hydroxyacetylene-diureine-carboxylic acid. This latter compound breaks down either to uroxic acid or to allantoin, depending on the hydrogen ion concentration of the solution from which the reaction product is crystallized. Hydroxyacetylene-diureine-carboxylic acid has actually been isolated in the form of an amorphous silver salt by Schuler and Reindel (2) as an intermediary oxidation product of uric acid.

The mechanism of oxidation of uric acid by enzymes extracted from mammalian tissues was first investigated by Wiechowski (3) in 1907. He demonstrated that allantoin was formed in the reaction by isolating it from the reaction mixture. In 1909 Battelli and Stern (4) in the course of a thorough investigation of the properties of uricase showed that the amounts of oxygen used and  $\text{CO}_2$  produced during this enzymatic oxidation agreed fairly well with those required by the theory that allantoin was the only product. However, in 1929 Felix and coworkers (5) published experiments indicating that under certain conditions  $\text{O}_2$  consumption proceeded at a faster rate than  $\text{CO}_2$  production and demonstrated the existence of two different optimal hydrogen ion concentrations for  $\text{O}_2$  consumption and  $\text{CO}_2$  production. They postulated the presence in their preparation of two separate enzymes catalyzing oxidation and decarboxylation. In 1932 Schuler (6) demonstrated that at pH 8.9 only 17.8 per cent of the theoretical amount of  $\text{CO}_2$  was formed and he produced some evidence that hydrox-

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yacetylene-diureine-carboxylic acid was formed during the enzymatic oxidation of uric acid. Keilin and Hartree (7), however, were unable to confirm the data of Felix and Schuler. They found only one pH optimum of 9.2 for the oxidation of uric acid and, without quoting specific experiments, stated that the respiratory quotient was consistent with the reaction which they formulated as follows:



Davidson (8) in 1942 used a highly purified enzyme preparation for the oxidation of uric acid and isolated allantoin from the reaction mixture. He therefore concluded that only one enzyme is involved in the formation of allantoin from uric acid. This is convincing evidence that allantoin is formed by the action of uricase. However, it will be shown in this paper that more than one end-product is formed in the reaction, presumably by spontaneous decomposition of an extremely unstable primary product of the enzymatic oxidation of uric acid.

### Methods

Uricase was prepared either from pig liver or beef kidney. No differences could be detected in the action of these two preparations. The tissues were ground and extracted with acetone according to the method of Keilin and Hartree (7). 4 gm. of the dried powder were extracted at 38° with 100 ml. of Sørensen's borate buffer of pH 9.2 and the clear extract brought to a pH of 4.5 by adding 0.46 part of 0.5 *N* acetic acid. The precipitate thus formed contained the entire enzyme activity which equaled approximately that of the ammonium sulfate precipitate of Davidson (8). The catalase content of the preparation was high. This acetic acid precipitate, prepared freshly and dissolved in borate buffer, was used in all experiments reported in this paper.

Uric acid was determined by the method of Folin (9).

O<sub>2</sub> consumption and CO<sub>2</sub> production were measured manometrically by Warburg's direct procedure, with vessels having two side arms.

The potassium salt of uroxanic acid was prepared by permanganate oxidation of uric acid according to Biltz's (10) directions. When prepared freshly, it contained 3 molecules of water. Some of the water was lost when the salt was stored in the desiccator. The tertiary silver salt of hydroxyacetylene-diureine-carboxylic acid was prepared according to the method of Schuler and Reindel (2).

### Results

The experiment shown in Fig. 1 demonstrates that during the oxidation of uric acid 0.5 equivalent of O<sub>2</sub> was used. This is in agreement with

Keilin's findings and demonstrates that  $\text{H}_2\text{O}_2$  formed in the reaction was completely decomposed by the action of catalase present in the enzyme preparation and that secondary oxidation due to  $\text{H}_2\text{O}_2$  did not take place. The amount of  $\text{CO}_2$  produced was only 40 per cent of that expected if the activity had proceeded according to Reaction 1. Fig. 1 also shows that the amount of  $\text{CO}_2$  does not increase after the completion of the oxidation. This is strong evidence against Schuler's theory that hydroxyacetylenediureine-carboxylic acid is formed by the action of uricase and then decarboxylated by a second enzyme. The findings can best be explained by the assumption that an extremely unstable primary oxidation product

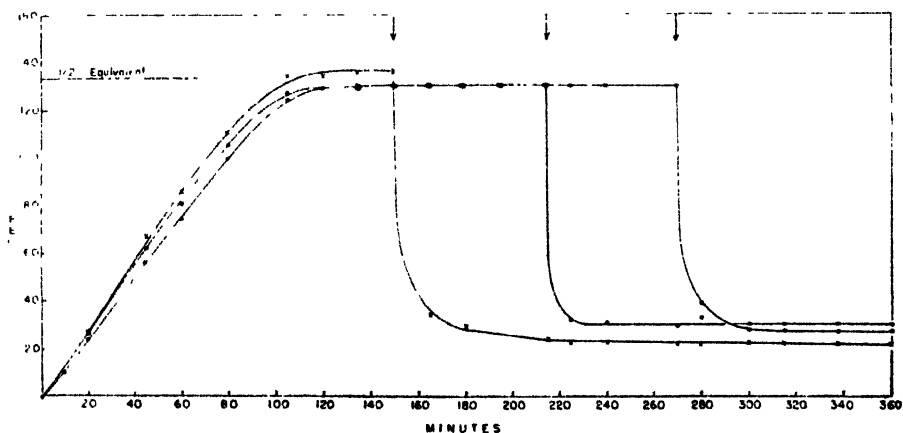


FIG. 1 Oxygen consumption and  $\text{CO}_2$  production during enzymatic oxidation of uric acid. Main vessel, 2 mg. of uric acid in 2 ml. of Sørensen's borate buffer, final pH 9.5; Side Vessel 1, 0.2 ml. of uricase solution corresponding to 0.12 gm. of acetone powder (beef kidney); Side Vessel 2, 0.3 ml. of 2 M lactic acid. Temperature  $38^\circ$ ; gas, oxygen. The three curves represent the results obtained from three separate vessels. At time zero, enzyme was added to the uric acid solution in all vessels. The arrows indicate the times of addition of lactic acid. Correction for  $\text{CO}_2$  contained in the reagents was applied.

is formed by the action of uricase which immediately decomposes with the formation of more than one stable compound. No changes in pressure occurred after the liberation of  $\text{CO}_2$  by lactic acid. This proves that the compounds formed are stable in weakly acid solution.

The isolation of allantoin (3.8) from the reaction mixture proves that this is the compound formed by oxidation and decarboxylation of uric acid, and the amount of allantoin formed is assumed to be equivalent to the quantity of  $\text{CO}_2$  produced during the reaction. Attempts to isolate other oxidation products failed. However, it was possible to identify these substances by indirect methods. The fact that they were formed from

uric acid without the loss of  $\text{CO}_2$  indicated that they contained 5 carbon atoms. They also contained all of the nitrogen originally present in uric acid, since neither ammonia nor urea was formed during the oxidation. This was shown by incubating uric acid with crude uricase in phosphate buffer at pH 8.0. After complete oxidation of uric acid, urea or ammonia could not be detected by the method of Van Slyke and Cullen (11). The use of borate buffer had to be avoided in this experiment because of its inhibitory effect on the action of urease.

The number of possible oxidation products of uric acid which contain 5 carbon and 4 nitrogen atoms is limited, and if the mode of linkage of the nitrogen atoms could be determined, the structure of the molecule would be known. The reaction with nitrous acid proved to be suitable for the solution of this problem. Biltz (10) had shown that in the presence of mineral acids nitrogen bound in ring form did not react with nitrous acid, while the nitrogen of an open chain was liberated. Thus uric acid yielded no nitrogen, while allantoin gave off 2 atoms. All 4 nitrogen atoms of uroxic acid were recovered. A micromethod was therefore worked out which permitted the determination of labile nitrogen in the high dilutions made necessary by the experimental conditions. Van Slyke's (12, 13) procedure for the determination of amino nitrogen, with acetic acid as the acidifying agent, was first attempted but proved unsuitable because of the slowness of the liberation of urea nitrogen and the relatively high blank values which occurred when the reaction was allowed to proceed for several hours. The use of dilute sulfuric acid, as recommended by Biltz (10), although allowing the reaction to proceed at a faster rate, was also accompanied by a high blank which made this method unsuitable for micro-determination. It was found that in the presence of lactic acid the nitrous acid reaction proceeded at a satisfactory speed, while the amount of nitrogen formed by the reagents alone was considerably smaller than in other procedures. Under these conditions, however, the amount of NO formed was too large to permit the use of the apparatus designed by Van Slyke. The apparatus shown in Fig. 2 obviated this difficulty and was satisfactory for our purpose. The reaction vessel, A, made from a 15 ml. centrifuge tube, was connected to a  $\text{CO}_2$  generator. In this reaction vessel 2 gm. of solid  $\text{NaNO}_2$  were placed, and 5 ml. of a neutralized solution containing 0.5 to 3 mg. of labile nitrogen were added. The vessel was then closed with a rubber stopper which contained the gas outlet as well as a burette (B) whose tip was drawn out to a fine capillary. This burette had previously been filled with concentrated lactic acid. The absorption tubes, C, contained a saturated solution of potassium dichromate in concentrated  $\text{HNO}_3$ . In order to assure complete absorption of NO, fresh reagent was used for each determination.  $\text{CO}_2$  was absorbed in tube D which contained

40 per cent KOH. The nitrogen was measured in a 5 ml. micro burette, *E*, over dilute alkali. The entire apparatus was freed of air by passing through it a current of air-free CO<sub>2</sub> and ejecting all gases collected in *D* and *E* through the 2-way stop-cock, *F*. 1 ml. of concentrated lactic acid was then added slowly from *B* and the solutions were mixed by passing a small amount of CO<sub>2</sub> through *A*. During this procedure, stop-cock *F* was kept closed and the leveling bulb connected to *D* was lowered sufficiently to cause slight negative pressure in the reaction vessel, *A*. The reaction was then allowed to proceed for exactly 1 hour, at the end of which time the stop-cock of *A* was opened and the nitrogen formed flushed from

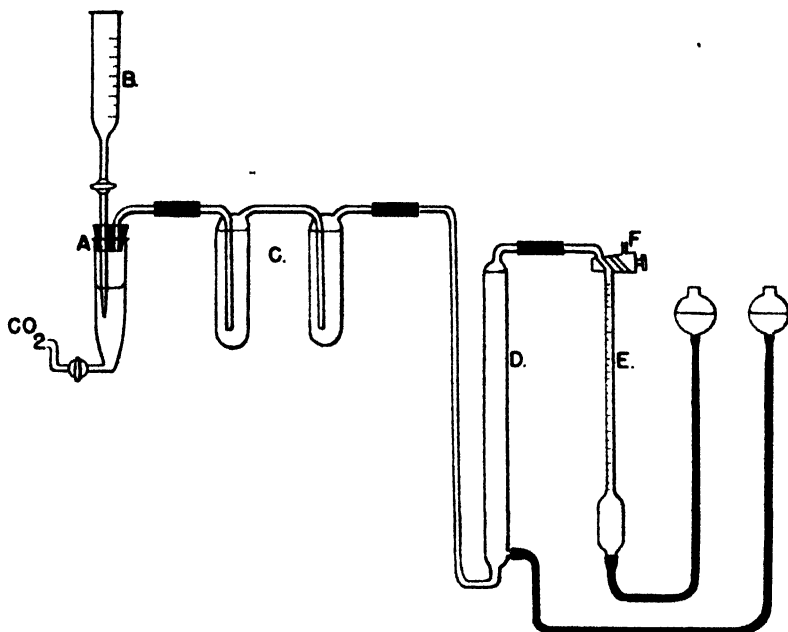


FIG. 2. Apparatus for the determination of labile nitrogen with nitrous acid

*A* into *D* by a rapid stream of CO<sub>2</sub>. During this procedure NO and CO<sub>2</sub> were absorbed. By means of the leveling bulbs, the nitrogen was then transferred to the burette, *E*, and measured. To test for completeness of absorption of NO, a modified Hempel pipette was attached to *F* and the gas treated with alkaline permanganate solution according to Van Slyke's directions. This procedure, however, proved to be unnecessary and was later abandoned, since absorption of NO by the dichromate solution was always complete. A blank determination was then run on 5 ml. of water. The amount of nitrogen formed from the reagents within 1 hour never exceeded 0.26 ml. The blank was subtracted from the experimental



reading and after the temperature and barometric pressure were measured the amount of nitrogen was calculated by means of the table given by Van Slyke (12).

In Table I the amounts of nitrogen liberated from various compounds at 25° during 1 hour are given. Only minimal amounts of nitrogen are formed from uric acid, since all of its nitrogen is bound in stable rings. Urea and uroxic acid on the other hand, which contain all nitrogen in open chains, react quantitatively with nitrous acid. The excess of nitrogen produced from uroxic acid is explained by the formation of malonic acid (10) which is known to liberate small amounts of nitrogen from nitrous acid (14). The urea nitrogen of allantoin reacts promptly, while the iminazole ring is fairly resistant to the action of nitrous acid. If, however, the reaction is permitted to proceed for longer periods, slightly more than 2 atoms of nitrogen are liberated. Hydroxyacetylene-diureine-carboxylic

TABLE I  
*Fraction of Nitrogen Which Reacts with Nitrous Acid*

	Total N	Labile N	Labile N Total N
	mg	mg	
Uric acid	1.67	0.066	0.04
Urea	2.26	2.25	1.00
K uroxic acid	1.73	1.88	1.09
Allantoin	2.87	1.21	0.42
Hydroxyacetylene-diureine-carboxylic acid* . . .	2.09	1.24	0.59

\* The tertiary silver salt was decomposed with an excess of NaCl and the filtrate from AgCl used for the determination. Total N was determined by the Kjeldahl method.

acid, an unstable compound, decomposes to allantoin in the presence of strong acids. This instability of its ring systems explains the liberation of 2 equivalents of nitrogen. There can be no doubt that the silver salt, which cannot be crystallized, contained appreciable amounts of uroxic acid and it is therefore not surprising that the amount of labile nitrogen obtained from this preparation is greater than that obtained from allantoin.

If two nitrogenous compounds are present in the reaction mixture, the total amount of nitrogen liberated by  $\text{HNO}_2$  is equal to the sum of the amounts of nitrogen given off by the substances individually. This was shown by analyzing 5 ml. of a solution containing 1.15 mg. of allantoin nitrogen and 1.04 mg. of uroxic acid nitrogen. The amount of nitrogen which reacted with nitrous acid in 1 hour was 1.65 mg. This is in agreement with the figure of 1.62 which is derived by adding the amounts of labile nitrogen of allantoin and uroxic acid calculated with the factors given in Table I.

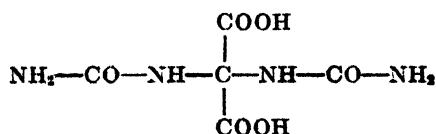
In the experiments summarized in Table II, uric acid was incubated with uricase in the presence of Sørensen's buffers in an atmosphere of  $O_2$  at  $38^\circ$ .  $O_2$  consumption and  $CO_2$  production were measured manometrically. After completion of the oxidation, proteins were precipitated with tungstic acid and 5 ml. of the neutralized filtrate analyzed for labile nitrogen by the method described above. In several experiments proteins were removed by ultrafiltration according to the procedure of Coolidge (15). The results obtained on ultrafiltrates agreed with those obtained on tungstate filtrates, indicating that tungstic acid did not cause decomposition of the oxidation products of uric acid.

In Experiment 1 borate buffer was used and the final pH of the solution was 9.10. From the amount of  $CO_2$  produced it is concluded that 0.27 equivalent of allantoin was formed from uric acid. Since in 1 hour 0.42 of allantoin nitrogen reacts with nitrous acid,  $0.27 \times 0.42 = 0.113$  equivalent

TABLE II  
*Distribution of Labile Nitrogen in Oxidation Products of Uric Acid*

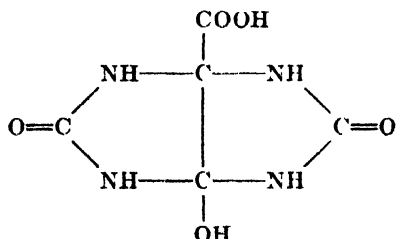
Experiment No. Buffer	1 Borate 9.10	2 Phos- phate 6.76	3 Phos- phate 7.93
pH			
$CO_2$ produced per mole uric acid, <i>mole</i>	0.27	0.54	0.55
Total N per ml filtrate, <i>mg</i>	0.320	0.389	0.251
Labile " " " " "	0.284	0.169	0.148
" " in terms of total N, <i>equivalent</i>	0.887	0.434	0.590
" " from allantoin, in terms of total N, <i>equivalent</i>	0.113	0.227	0.231
" non-allantoin N, " " " " " "	0.774	0.207	0.359
Total " " " " " " " "	0.73	0.46	0.45
Labile			
Total non-allantoin N	1.06	0.45	0.80

of the labile nitrogen is accounted for by allantoin. The remaining 0.774 equivalent of labile nitrogen must have been liberated from a compound containing 0.73 of the total nitrogen present. This proves that in addition to allantoin a substance was formed which on reaction with nitrous acid loses 4 nitrogen atoms which must therefore be linked in open chains. This substance has also been shown to contain all carbon originally present in uric acid and to be stable in weakly acid solution. The only oxidation product of uric acid which conforms to this description is uroxic acid.



In the second column of Table II the results of an experiment are summarized in which the oxidation of uric acid was carried out in  $m/15$  phos-

phate buffer at pH 6.76. These results indicate that the amount of allantoin formed is larger than that which was formed in alkaline borate buffer. The yield of labile nitrogen of only 0.434 equivalent indicates that in addition to allantoin a compound was formed which contained 0.45 equivalent of its total nitrogen or 2 of its 4 nitrogen atoms in a labile linkage. Although more than one theoretical possibility could explain these results, it appears most probable that hydroxyacetylene-diureine-carboxylic acid was formed, because this is the only known oxidation product of uric acid which contains 5 carbon atoms and 4 nitrogen atoms, of which 2 are lost on treatment with nitrous acid.



The figure of 0.45 for the equivalent of labile nitrogen in hydroxyacetylene-diureine-carboxylic acid obtained from this experiment appears more significant than that given in Table I, which was obtained from an impure compound. Although subject to slight error, it will be used in subsequent calculations.

In Experiment 3 given in Table II uric acid was oxidized in phosphate buffer at pH 7.93. The results indicate that the labile nitrogen in the non-allantoin fraction amounted to 0.80 of its total nitrogen. This figure can be explained only by the assumption that the non-allantoin fraction was composed of two compounds; namely, uroxic acid and hydroxyacetylene-diureine-carboxylic acid.

In Table III are given the equivalents of labile nitrogen and  $\text{C}^1\text{O}_2$  formed per molecule of uric acid by the action of uricase at various hydrogen ion concentrations and in different buffer solutions. The amounts of hydroxyacetylene-diureine-carboxylic acid and uroxic acid were calculated from the ratios of labile to total nitrogen in the non-allantoin fraction, determined as above, and the ratios of labile to total nitrogen in hydroxyacetylene-diureine-carboxylic acid and uroxic acid given in Table I. Table III demonstrates that the relative quantities of the three reaction products vary not only with the concentration of hydrogen ions but are also a specific function of the buffer ions. Change in oxygen tension or concentration of enzyme had no effect on the composition of the oxidation products. Similarly, identical results were obtained whether the enzyme was prepared from beef kidney or from pig liver.

The stability of the oxidation products of uric acid in the presence of the enzyme preparation is sufficient proof that these do not represent successive steps of degradation. However, the possibility that each of these compounds is the result of the simultaneous action of a different enzyme deserves consideration. If this were the case, the allantoin formed would exhibit optical activity, since this is a property common to all known enzymatic products containing asymmetric carbon. The following experiment demonstrated that allantoin formed by the action of uric acid is racemic. Uric acid, dissolved in phosphate buffer of pH 7.45, was incubated with uricase. At the end of 2 hours, 1.28 mg. of uric acid per ml. were oxidized. After precipitation of proteins with tungstic acid, causing a dilution of 10 per cent, no optical rotation was detected in a 200 mm. tube. The optical activity of *d*-allantoin according to Thomas and DeGraeve (16) is  $\alpha_D = +93^\circ$ . Since under the conditions of the experiment

TABLE III  
Relative Quantities of Oxidation Products of Uric Acid

Buffer pH	P 6.76	P 7.31	P 7.93	B-HCl 7.87	B 9.10	B-NaOH 9.45	G 9.14
Equivalent labile N $\times$ 0.25	0.434	0.553	0.590	0.700	0.887	0.715	0.78
CO <sub>2</sub> (allantoin)	0.54	0.57	0.55	0.09	0.27	0.40	
HDC	0.46	0.24	0.21	0.52	0.03	0.17	
uroxanic acid	0	0.19	0.24	0.39	0.70	0.43	

HDC = hydroxyacetylene-diureine-carboxylic acid; P = Sørensen's phosphate mixture, B-HCl = Sørensen's borate-HCl mixture; B = Sørensen's standard borate; B-NaOH = Sørensen's borate-NaOH mixture, G = Sørensen's glycine-NaCl-NaOH mixture.

55 per cent of uric acid is converted to allantoin, a rotation of  $0.12^\circ$  should have been found if the allantoin had been optically active. According to Fosse *et al.* (17) optically active allantoin is stable in neutral and acid solutions and therefore racemization did not occur during the experiment.

#### DISCUSSION

According to the findings reported in this paper, the breakdown of uric acid by uricase *in vitro* must be rewritten as follows:

- (2)  $\text{Uric acid} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{C}_5\text{N}_4\text{O}_4\text{H}_4 + \text{H}_2\text{O}_2$   
 $\rightarrow (a) + \text{H}_2\text{O} \rightarrow \text{hydroxyacetylene-diureine-carboxylic acid}$   
 (3)  $\text{C}_5\text{N}_4\text{O}_4\text{H}_4 \rightarrow (b) + \text{H}_2\text{O} \rightarrow \text{allantoin} + \text{CO}_2$   
 $\rightarrow (c) + 2\text{H}_2\text{O} \rightarrow \text{uroxanic acid}$

Reaction 2 is catalyzed by uricase. The primary reaction product is unknown and may have a higher water content than is indicated by the

formula given. This compound is unstable and decomposes in all probability without intervention of enzymes according to Reaction 3. The ratio of the products formed varies with the conditions of the experiment, reaction (a) being enhanced in neutral or slightly acid medium, while reaction (b) or (c) prevails in alkaline buffers. A distinct inhibitory effect of borate ions on the formation of allantoin, reaction (b), is evident from the comparison of the  $\text{CO}_2$  production in the presence of borate and glycine buffers. This effect might be attributable to the well known affinity of borate ions to adjacent hydroxy groups. In order to explain an inhibition of decarboxylation, these hydroxy groups would have to be located in 5 and 6 positions, thus suggesting that oxidation of the double bond of uric acid is the primary enzymatic reaction. The fact that allantoin production is considerably higher in the absence of borate explains the high yields of  $\text{CO}_2$  reported by Battelli and Stern (4) who used ammonium buffers in their experiments. Whether Reaction 3 occurs *in vivo* is unknown. Neither uroxic acid nor hydroxyacetylene-diureine-carboxylic acid has been found so far in urine. Optically active allantoin has been isolated from cattle urine by Thomas and DeGraeve (16) and it would therefore appear likely that *in vivo* the product of uricase action is, at least in part, converted to allantoin by an enzyme which as yet has not been isolated.

#### SUMMARY

The amount of  $\text{CO}_2$  formed during enzymatic oxidation of uric acid is only a fraction of 1 equivalent, indicating that substances other than allantoin are formed in the reaction. From the amount of nitrogen which these compounds lost on treatment with nitrous acid, it was concluded that these substances are uroxic acid and hydroxyacetylene-diureine-carboxylic acid. All these compounds are stable in the presence of uricase. The primary product of uricase action is an unstable compound which, without intervention of enzymes, decomposes into the three reaction products. The relative amount of these products formed depends not only on the concentration of hydrogen ions but also on the buffers used.

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# THE ESTIMATION OF SMALL AMOUNTS OF CARBON MONOXIDE IN BLOOD\*

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During the past 3 years, Roughton and Horvath (1, 2) have described some improvements in the gasometric estimation of carbon monoxide in blood with the Van Slyke apparatus. Recently Scholander and Roughton (3) have applied their syringe-capillary method to the problem and have attained an accuracy of the same order as that of many of the Van Slyke methods. In "Appendix II" of their paper Roughton described a combination of the Van Slyke and the syringe-capillary techniques (see also (4)). Further development of the latter method since 1943 has shown that it is possible to estimate the CO liberated from blood by vacuum and acid ferri-cyanide with an average accuracy of about  $\pm 0.10$  c.mm. if the total CO does not exceed 10 c.mm.; i.e., to within  $\pm 0.02$  volume per cent in the case of 0.5 cc. blood samples of CO content ranging from 0 to 2 volumes per cent. To reach such a standard with previous Van Slyke gasometric methods has required blood samples of at least 2 cc., and the present method has therefore proved of value in CO-blood volume determinations, especially in the case of small animals from which only limited quantities of blood can be drawn. The method has also been applied to the estimation of low percentages of CO in air (see the accompanying paper (5)) and to the study of the fate of CO in the body during recovery from carbon monoxide poisoning.

With larger volumes of liberated CO, i.e. 10 to 200 c.mm., the average accuracy ranges correspondingly from 0.15 to 0.40 c.mm. These figures are on a par with the most accurate O<sub>2</sub> content determinations quoted by Peters and Van Slyke (6). It has often been difficult, hitherto, to obtain quite such good results in the case of most gasometric CO methods. The present technique therefore has advantages over the whole range of CO saturation, though our experience of it has been most extensive in the lower range.

The full experimental details and tests of the combined Van Slyke-syringe method are given below. Some of the features have been described before, but, since lack of attention to any single detail in the technique is liable to lead to a loss of accuracy, it has seemed well to give a complete résumé of all details of the method, old and new.

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.



## EXPERIMENTAL

*Principle of the Method*—The blood is laked and shaken twice *in vacuo* in the Van Slyke-Neill gasometric apparatus with a sodium borate-hydrosulfite solution. The dissolved nitrogen is completely extracted, the CO, O<sub>2</sub>, and most of the CO<sub>2</sub> remaining in solution chemically bound. The extracted N<sub>2</sub> is quantitatively ejected without loss of solution, and the CO and CO<sub>2</sub> of the solution are then liberated by shaking with a deaerated ferri-cyanide-acetate mixture, the O<sub>2</sub> remaining bound by the hydrosulfite. After absorption of the CO<sub>2</sub> by deaerated alkali, the pressure of the residual gas (*i.e.*, CO plus a trace of N<sub>2</sub>) is measured at the 2.0 and 0.5 cc. marks, and the percentage of CO therein is obtained by transferring the whole, or an aliquot, of the bubble from the Van Slyke chamber to the Scholander-Roughton syringe-capillary apparatus, where it is accurately analyzed according to their usual procedure. From these readings the CO content of the blood sample is readily calculated.

*Reagents*—

Caprylic alcohol.

1 per cent saponin. This is freshly prepared each day. The "toxic" brand of Eimer and Amend has proved quite satisfactory.

3 per cent sodium borate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ).

Solid  $\text{Na}_2\text{S}_2\text{O}_4$ .

32 per cent  $\text{K}_3\text{FeCN}_6$  (analytical grade).

Acetate buffer. 70 gm. of sodium acetate ( $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ ) are dissolved in 100 gm. of water and 15 cc. of glacial acetic acid are added.

Winkler's solution. 20 gm. of cuprous chloride, 25 gm. of ammonium chloride, and 75 gm. of water are placed in a bottle just large enough to hold them. The bottle is corked, shaken with as little air as possible, and the precipitate then allowed to settle. A coil of copper wire is placed in the solution, which is then covered with a layer of paraffin oil. After some time the reagent becomes colorless.

30 per cent NaCl.

4 per cent NaOH. 50 to 70 cc. are stored in a 100 cc. burette with rubber connections and pinch-cocks at each end.

45 per cent urea.

Roughly 2 N HCl. 1 part of concentrated HCl is diluted with 4 parts of distilled water.

Hydrosulfite-borate solution. This is prepared each day by filling a 25 cc. test-tube almost to the top with a 3 per cent sodium borate solution and then tipping in roughly 0.5 gm. of sodium hydrosulfite. The tube is corked and shaken to dissolve the  $\text{Na}_2\text{S}_2\text{O}_4$ , and the mixture is then poured into a 25 cc. burette with minimum air contact.

Every 2 or 3 days 1 part of acetate buffer is mixed with 4 parts of 32 per

cent  $K_3FeCN_6$ , and 50 to 70 cc. of the mixture placed in a 100 cc. burette with rubber connections and pinch-cocks at each end.

The NaOH solution and the ferricyanide-acetate solution are deaerated in their respective burettes which are connected to a water pump, evacuated, and shaken for 2 minutes. The burettes are placed vertically in stands and the vacuum released by opening the top pinch-cock shortly before the solutions are required in the analysis. No oil is used in the burettes in view of the high solubility of air in oil and the possibility of traces of oil sticking to the sides of the burette, thereby re-aerating the solutions to a slight extent. In the present technique it is essential to keep the dissolved air content of the NaOH and ferricyanide at a minimum, and for best results the burettes should be re-evacuated and shaken every hour during the course of the day's measurements. Alternatively the NaOH may be stored over mercury in a Sendroy vessel (7), but this is undesirable in the case of the ferricyanide solution because of the oxidizing action of the latter on the metal.

#### *Procedure*

5 cc. of distilled water are first evacuated and shaken in the clean chamber of the Van Slyke apparatus, and the mercury reservoir then lowered so as to draw the evacuated water well down into the rubber connection between the chamber and the rest of the apparatus. The evacuated water and liberated air are then expelled and 3 to 4 drops of caprylic alcohol drawn into the chamber. 0.5 to 2.0 cc. of the blood, the CO content of which is to be analyzed, are then run into the Van Slyke cup and thence into the chamber. 2 cc. of 1 per cent saponin are next put into the cup and run slowly into the chamber, the tap closed, and the blood and the saponin mixed and allowed to stand for about a minute to insure complete laking. 2 cc. of the  $Na_2S_2O_4$ -borate solution are then run into the cup and thence into the chamber; the saponin and the  $Na_2S_2O_4$  solution are sufficient to wash all the blood quantitatively into the chamber. This method of introducing the blood into the chamber is feasible in instances such as the present in which slight contact of the blood with air does not matter, and we have found it more convenient than the usual one of delivering the blood from a rubber-tipped pipette through solution in the cup, though the latter can be adhered to if the reader prefers.

The tap is sealed and the mercury is lowered to the 50 cc. mark, the chamber covered with black paper, and the blood solution evacuated by shaking for  $1\frac{1}{2}$  minutes. The extracted gases are quantitatively expelled without loss of blood solution and the evacuation repeated for  $1\frac{1}{2}$  minutes to insure complete extraction of the dissolved  $N_2$ . The small bubble obtained even after the second extraction is mainly  $CO_2$ . The cup is dried with a roll of

filter paper so that the deaerated solution of ferricyanide is not contaminated with traces of air-containing water when it is run into the cup. 3 to 4 cc. of the ferricyanide are cautiously run into the cup, and the lower 1 cc. thereof drawn into the chamber. The tap is sealed and the solution shaken for 3 minutes at the 50 cc. mark, after which the  $\text{CO}_2$  is absorbed from the extracted gas by adding 1.5 cc. of deaerated 4 per cent NaOH from the cup in the usual way. To guard against admission of any dissolved air at this stage, the cup is again dried before the deaerated NaOH is run in (3.5 to 4.0 cc. of the latter are placed in the cup). The residual gas in the chamber is then compressed nearly to atmospheric pressure, thus mixing the blood solution thoroughly with the films of 4 per cent NaOH remaining on the upper part of the chamber (these have an appreciably lower aqueous vapor pressure than the blood solution). The pressure reading is then taken in the ordinary way at the 2.0 cc. mark ( $p_{12}$ ), at least 1 minute being allowed for drainage, and also at the 0.5 cc. mark ( $p_{1.5}$ ).<sup>1</sup>

The next step is to determine the percentage of CO in this gas in the chamber. This is done in the following way. The Van Slyke cup is filled with 30 per cent NaCl and the gas in the chamber brought up nearly to atmospheric pressure. The tap is opened cautiously to allow a drop or two of NaCl solution to pass through and clear the mercury from the capillary bore of the tap. The tap is then closed and a Scholander-Roughton syringe-capillary apparatus, previously filled with 30 per cent NaCl solution, is inverted and its cup pressed fairly snugly against the bottom of the Van Slyke cup. It is important that the salt solution in the syringe-capillary be quite free of air bubbles. The mercury reservoir of the Van Slyke apparatus is then raised so as to put the gas bubble at the top of the chamber under slight positive pressure. The lower tap is closed and the upper tap is then cautiously opened so that the gas bubble expands through the bore of the tap. By suitably squeezing the chamber down onto its lower rubber connection, 10 c.mm. or so of the bubble can be coaxed up into the inverted cup of the syringe-capillary, from which it is readily withdrawn into the capillary by manipulation of the plunger of the syringe. If the bubble is too big for the capillary, the excess is ejected. The syringe is then placed in a water bath (e.g., a 1000 cc. beaker), the rest of the gas quantitatively expelled to the air from the Van Slyke chamber without loss of any solution, and the manometer readings taken at the 2 cc. mark ( $p_{22}$ ) and the 0.5 cc.

<sup>1</sup> During the admission of the NaOH the blood precipitate usually redissolves almost completely. Occasionally, however, appreciable traces remain behind on the walls of the chamber between the 0.5 and 2.0 marks and readings at the 2.0 cc. mark should then be disregarded. The part of the chamber above the 0.5 cc. mark is quite clean in almost every case, so that the validity of the 0.5 cc. mark readings is not affected.

mark ( $p_{2.0}$ ) without any gas in the chamber. The blood mixture is then ejected from the chamber, and the latter is filled three-fourths full with water to which 2 or 3 cc. of 2 per cent  $\text{Na}_2\text{S}_2\text{O}_4$  in 4 per cent  $\text{NaOH}$  have been added. The mercury is set at the 50 cc. mark and the chamber shaken gently while the analysis of the bubble is being completed in the syringe-capillary. This takes 3 to 4 minutes, by which time the remnants of blood precipitates in the Van Slyke chamber are usually completely dissolved. Occasionally the chamber may be left overnight full of 45 per cent urea, which is very effective in dissolving obstinate blood precipitates.

The analysis of the bubble for CO is carried out according to the directions of Scholander and Roughton (3). If  $l_1$  is the length of the bubble in capillary divisions before absorption with Winkler's solution, and  $l_2$  is the length of the bubble after completion of the absorption, then

$$100 \times (l_1 - l_2)/l_1 = \% \text{ CO in the bubble} \quad (1)$$

$$100 \times l_2/l_1 = \% \text{ N}_2 \text{ in the bubble} \quad (2)$$

The CO content of the blood then equals

$$(p_{12} - p_{22}) \times \frac{l_1 - l_2}{l_1} \times f_2 \text{ volumes } \% \quad (3)$$

or

$$(p_{1.0} - p_{0.5}) \times \frac{l_1 - l_2}{l_1} \times f_{0.5} \text{ volumes } \% \quad (4)$$

in which  $f_2, f_{0.5}$  are the factors for the quantity of blood used at 2 cc. and 0.5 cc. marks respectively. These are read off from the appropriate columns in Table 30 of Peters and Van Slyke (6).

The syringe is cleaned by rinsing first with 2 N HCl and then with water. The Van Slyke chamber is also rinsed several times with water after expulsion of the alkaline cleaning fluid, and is then ready for the next analysis. The complete cleansing procedure, if applied in every determination, keeps the mercury and the chamber beautifully clean and adds, we believe, to the accuracy of the analyses.

If  $p_{12} - p_{22}$  is greater than 20 mm. of Hg, the CO content of the blood is taken to be the mean of the values given by the readings at the 2.0 and the 0.5 cc. marks, whereas if  $p_{12} - p_{22}$  is less than 20 mm. of Hg and greater than 4 mm. of Hg, extra weight is given to the 0.5 cc. readings in the proportion of 2 to 1. If  $p_{12} - p_{22}$  is less than 4 mm., it is not worth taking readings at the 2.0 cc. mark; on the other hand the size of the residual bubble of  $\text{CO} + \text{N}_2$  is now so small (less than 10 c.mm.) that the whole of it can be quantitatively transferred to the syringe apparatus and measured in the capillary. This gives an alternative duplicate analysis, which we believe

to be the most accurate method for blood containing very low amounts of CO; *e.g.*, for 2 cc. samples of blank blood of non-smokers and laboratory animals or for 0.5 cc. samples containing less than 2 volumes per cent CO. The calculation of the CO content of the blood in this case is as follows.

The volume of CO obtained in the capillary =  $(l_1 - l_2) \times$  the volume of each division of the capillary  $\times f$ , where  $f$  is the factor for converting to 0° and 760 mm. of Hg from the temperature of the water bath and the existing barometric pressure.  $f$  may be read off from Table 15, p. 129, of Peters and Van Slyke (6),

$$= (l_1 - l_2) \times 0.393 \text{ c.mm.} \times f$$

If  $S$  = the volume of blood sample used in cc., the CO content of the blood equals

$$(l_1 - l_2) \times f \times 0.0393/S \text{ volumes } \% \quad (5)$$

It is, of course, essential that the complete gas bubble be transferred quantitatively from the Van Slyke chamber to the syringe apparatus without any loss.<sup>2</sup> The bubble must also be gotten into the capillary as smoothly and expeditiously as possible; otherwise some gas exchange with the air dissolved in the saturated NaCl solution may take place.

It will be noted that the method contains no  $c$  correction, and therefore in blank experiments with 2 cc. of water in place of blood  $l_1 - l_2$  should be 0. In two such tests, by using freshly deaerated ferricyanide and NaOH,  $l_1 - l_2$  was found to be 0.2 and 0.25 capillary divisions respectively, *i.e.* 0.075 c.mm. of gas; this would cause an error in the CO content of only 0.015 volume per cent with a 0.5 cc. blood sample, and correspondingly smaller errors with larger blood samples. By using ferricyanide and NaOH solution, which had been deaerated some hours previously and had stood at rather a low level in their respective burettes, larger values of  $l_1 - l_2$  were found; namely, 0.5 and 0.45 divisions. Undoubtedly the solutions had absorbed some air, and since Winkler's solution absorbs O<sub>2</sub> as well as CO, a perceptible increase in  $l_1 - l_2$  occurred. It is for this reason indeed that so much care must be exercised in preventing any admission of dissolved air into the chamber, once the evacuation with borate-hydrosulfite is finished; as an alternative the introduction of an oxygen absorbent in place of NaOH was considered, but this was found to cause more trouble than the exercise of the precaution mentioned above.

From Equations 3 and 4 it follows that the residual N<sub>2</sub> in the gas extracted in the Van Slyke chamber by the ferricyanide should exert a pressure at the

<sup>2</sup> The observer should therefore be careful to select a properly constructed chamber with the upper part converging to the bore of the top tap without any intervening shelf. The bore of the tap and the glass passages on either side must also be scrupulously free from grease and dirt.

2.0 cc. mark of  $(p_{12} - p_{22}) \times l_2 / l_1$ . In properly conducted experiments with freshly deaerated solutions this pressure should lie within the range of 0.4 to 0.8 mm.; it should be, in fact, equivalent to the  $c$  correction in Horvath and Roughton's method. Whether it does, in fact, lie within these limits provides conversely a check as to the correct conduct of the analysis.

### *Tests and Accuracy of the Method*

*CO Contents Ranging from 0 to 2 Volumes Per Cent*—Table I shows the results of a carefully conducted series of tests in which known mixtures of CO-saturated blood and blank blood, *i.e.* normal blood drawn directly from man or the dog, were analyzed. The expected CO content is calculated from the measured CO contents of the saturated and the blank blood, and the proportion in which these were mixed. The latter was so accurately

TABLE I  
*Analysis of 0.05 Cc. Blood Samples with CO Contents of 0 to 2 Volumes Per Cent;  
Comparison of Observed and Expected Values*

Observed CO content	Expected CO content $\pm 0.02$	Discrepancy
<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>
0.47	0.46	+0.01
0.79	0.83	-0.04
0.80	0.80	0.00
0.82	0.83	-0.01
0.87	0.86	+0.01
0.90	0.87	+0.03
1.05	1.03	+0.02
1.21	1.19	+0.02
1.32	1.34	-0.02
1.52	1.51	+0.01

determined, either by pipetting or by weighing, that error arising from any uncertainty in it can be neglected; the measured CO contents of the saturated blood and the blank blood are, however, subject to possible errors of  $\pm 0.05$  and  $\pm 0.01$  to  $\pm 0.02$  volume per cent respectively. The calculation of the CO content of the mixtures of the two (proportions ranging from 1 to 10 up to 1 to 40) has therefore an over-all uncertainty of  $\pm 0.015$  to  $\pm 0.02$  volume per cent.

All the tests in Table I were done with 0.5 cc. samples of the blood mixtures since, as mentioned in the introduction, it was desired to see whether, with the present method, an accuracy comparable to that obtained in previous methods with 2.0 to 5.0 cc. blood samples could be reached. In eight out of the ten tests given in Table I, the discrepancy between observed and expected CO contents was only 0.02 volume per cent or less; in the remain-

ing two the discrepancy was 0.03 and 0.04 volume per cent respectively. The average discrepancy was  $\pm 0.017$  volume per cent, there being no appreciable systematic effects. In view of the uncertainty of  $\pm 0.02$  volume per cent in the expected CO contents it seems reasonable to conclude that the observed CO contents are in general accurate to  $\pm 0.02$  volume per cent within the range tested, *i.e.* 0.0 to 2.0 volumes per cent, or to within  $\pm 0.10$  c.mm., since 0.5 cc. blood samples were used. The latter figure corresponds to about 0.3 division on the syringe-capillary.

Six tests were also done with larger blood samples; *i.e.*, 1.0 or 2.0 cc. The accuracy obtained was at least as good as, and possibly better than, that with the 0.5 cc. samples, but the results are not numerous enough to be sure on the latter point.

In a second set of tests, given in Table II, known small volumes of CO-saturated blood were delivered into the cup of the Van Slyke apparatus and promptly washed into the chamber quantitatively with a few cc. of 1 per

TABLE II  
*Comparison of Observed and Expected CO Contents of Blood-Saline Mixtures*

Observed volume of CO	Expected volume ( $\pm 0.05$ ) of CO	Discrepancy
c. mm.	c. mm.	c. mm.
2.32	2.51	0.19
3.98	4.02	0.04
5.54	5.39	0.15
6.24	6.33	0.09
6.57	6.75	0.18
8.13	8.04	0.09

cent NaCl solution. The CO content of the blood-saline mixture was then determined (in c.mm.) and compared with that to be expected from the volume of CO-saturated blood pipetted into the cup. For blood volumes of 0.2 cc. and over the usual Van Slyke pipettes were used; the smaller volumes of blood were carefully measured out from a Scholander micro pipette (8). The object of these tests was to eliminate the major uncertainty in the former set; *i.e.*, that caused by the CO content of the blank blood. In this way it was possible to keep the uncertainty in the expected CO content as low as  $\pm 0.05$  c.mm. as long as the total content was not greater than 10 c.mm. The average discrepancy found in the lower range was  $\pm 0.12$  c.mm., and the maximum 0.19 c.mm.; allowing for an uncertainty of 0.05 c.mm. in the expected value, this indicates an average absolute accuracy of 0.05 to  $\pm 0.15$  c.mm., which is on a par with that reached in the results given in Table I.

A further check of the reliability of the technique for low CO contents was

obtained by comparing the results calculated from the readings at the 0.5 cc. mark in the Van Slyke chamber with those calculated from the readings in the capillary of the Scholander-Roughton apparatus. In twenty-six recent analyses of 1 or 2 cc. samples of blank bloods (CO contents ranging from 0.05 to 0.30 volume per cent), the figures tallied to within  $\pm 0.01$  volume per cent on the average, the maximum discrepancy (save in two dubious experiments) being  $\pm 0.02$  volume per cent. Provided the bubble is completely transferred from the Van Slyke chamber to the capillary, as can usually be checked by eye, there is indeed no systematic discrepancy between the two methods, the differences being as often in one direction as in the other.

Scholander and Roughton (3), using the syringe-capillary technique alone, were able to obtain in the case of blood of low CO content an accuracy of about 0.03 to 0.05 volume per cent in the case of 0.12 cc. blood samples. With samples 4 times as large, viz. 0.5 cc. as in the present paper, an accuracy of 0.012 volume per cent should therefore be obtainable if the total amount of CO can be accommodated in the capillary of the syringe. This accuracy is almost but not quite reached in the present procedure in which the Van Slyke and syringe-capillary techniques are combined; any falling short is probably due to the number and complexity of the various steps in the method. To simplify the latter, we have it in mind to attach the usual capillary of the Scholander-Roughton apparatus to a 10 cc. instead of a 1 cc. syringe. With this device and with sundry small changes in the technique, it should be possible to apply the Scholander-Roughton principles to 0.5 cc. blood samples, and thus it is hoped to attain the same or greater accuracy than in the technique of this paper, but with much more ease and speed and without the need of a Van Slyke apparatus at all. Owing to pre-occupation with other work we have not yet been able to put this project to practical test.

*CO Contents Ranging from 2.0 to 5.0 Volumes Per Cent*—A smaller number of tests were carried out in this range. The results are given in Table III. With the 0.5 cc. samples of whole blood, Experiment A, the discrepancy between observed and expected values is less than might be expected, for in none of the four cases does it exceed 0.03 volume per cent, or 0.15 c.mm. when expressed as total volume of CO liberated. In the blood-saline mixtures, Experiment B, the discrepancy is somewhat higher, running up to 0.30 c.mm. in the last example. The average discrepancy for all seven results of Table III is  $\pm 0.16$  c.mm., corresponding to  $\pm 0.03$  volume per cent with 0.5 cc. blood samples. This is the same as that reported by Peters and Van Slyke ((6) p. 321) in a specially accurate set of  $O_2$  content determinations, and corresponds to an allowable error of  $\pm 0.25$  mm. of Hg in the reading of the pressure difference at the 0.5 cc. mark. This degree of



accuracy is adequate for all present practical purposes within this range of blood saturation, including of course the determinations of blood volume by the CO method.

*CO Capacities (CO Contents of 16 to 24 Volumes Per Cent)*—In fifteen typical pairs of determinations, with 1 cc. blood samples, the differences between duplicates were 0.01, 0.01, 0.02, 0.02, 0.02, 0.02, 0.03, 0.05, 0.05, 0.05, 0.05, 0.07, 0.07, 0.08, and 0.11 volume per cent with an average of 0.04 volume per cent, indicating a somewhat better precision than that obtained by Horvath and Roughton (2). The last figure corresponds to a pressure difference of 0.16 mm. of Hg in the Van Slyke apparatus if readings are taken at the 2.0 cc. mark. With readings at the 0.5 cc. mark the total

TABLE III  
*Comparison of Observed and Expected CO Contents*

Experiment A 0.05 cc. mixtures of CO-saturated blood and blank blood; range 2 to 5 volumes per cent		
Observed CO content	Expected CO content ( $\pm 0.02$ )	Discrepancy
<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>
2.06	2.05	+0.01
3.13	3.13	0.00
3.62	3.65	0.03
5.33	5.35	0.02
Experiment B. Mixtures of CO-saturated blood and saline, total CO volume 10 to 25 c mm.		
Observed volume of CO	Expected volume of CO	Discrepancy
<i>c mm.</i>	<i>c mm.</i>	<i>c mm.</i>
17.70	17.90 ( $\pm 0.10$ )	0.20
24.80	25.00 ( $\pm 0.15$ )	0.20
25.30	25.00 ( $\pm 0.15$ )	0.30

pressure difference would run up as high as 400 mm. of Hg, but since the distance between the 0.5 cc. mark and the top of the chamber averages 40 mm., an error of only 0.1 mm. in the setting of the liquid meniscus at the 0.5 cc. mark during the first pressure reading would lead to an error of 1 in 400 in the calculated CO content; *i.e.*, of 0.05 volume per cent in blood of average CO capacity. Thus whichever reading mark was used, the agreement between duplicates in the above set was as good as could be expected from the limitations of the human eye.

#### DISCUSSION

The main advantages of the combined Van Slyke-syringe technique described in this paper have been seen to be that (a) in the low range of CO

saturation an accuracy of  $\pm 0.02$  volume per cent is obtainable with much less blood than has hitherto been required to reach this standard; (b) in the upper range the accuracy is the maximum of which Van Slyke methods are capable, being limited only by the precision with which the liquid levels could be set and read. Other sources of error must therefore have been reduced to minor significance. In the present technique this is probably due to (a) the special pains to keep the Van Slyke chamber clean, (b) the fact that the liquid in the chamber is the same at the two critical pressure readings, so that errors due to variations in the vapor pressure, surface tension, etc., are automatically ruled out, (c) the lack of any *c* correction. Since *c* corrections can scarcely be read to better than 0.1 to 0.2 mm. of Hg, they do add appreciably to the uncertainty of the final figures when the Van Slyke methods are used under conditions of maximum accuracy with small amounts of gas.

The same combined procedure can also be applied to determinations of  $O_2$  content if the  $O_2$ - $N_2$  mixture, remaining in the chamber after the customary absorption of  $CO_2$  by soda, is transferred to the syringe-capillary and treated with alkaline hydrosulfite solution instead of Winkler's solution. Such technique might be of service when accurate determination of low  $O_2$  contents in small blood samples is required.

#### SUMMARY

A combination of Horvath and Roughton's modified Van Slyke method with the syringe-capillary technique of Scholander and Roughton is described. With this procedure the CO content of 0.5 cc. blood samples can be determined to within 0.02 volume per cent over the range 0 to 2 volumes per cent, and to within 0.03 volume per cent over the range 2.0 to 5.0 volumes per cent. The method is thus specially applicable to blood volume determinations on small animals, and is also of value in the high range of CO saturation, wherein the maximum accuracy of the Van Slyke technique is readily obtained.

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# THE ESTIMATION OF SMALL AMOUNTS OF CARBON MONOXIDE IN AIR\*

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Need has arisen lately for more precise methods of estimating CO in air, alike in the determination of blood volume, in the study of the fate of CO in the body, and in certain industrial and military problems related to CO. It has indeed become desirable to measure 0.005 to 1.0 volume per cent of CO in air to within 1 to 2 per cent of the amount determined. The advantages of using blood as the "active reagent" have been pointed out by Sendroy (1). Two general types of method have so far been available. In the first of these, used extensively by Haldane (2), the CO-air mixture is shaken in a tonometer with a few cc. of diluted blood solution until equilibrium is reached, and the per cent COHb in the solution is then determined colorimetrically or spectrometrically. The ratio of CO to O<sub>2</sub> in the gas mixture is then calculated from the equation

$$\% \text{ CO in gas} = \frac{\% \text{ O}_2 \text{ in gas}}{M} \times \frac{\% \text{ COHb}}{\% \text{ O}_2\text{Hb}} \quad (1)$$

The partition coefficient  $M$  is determined with the aid of known CO-air mixtures; its value usually lies between 200 and 300. Allowance must also be made for the amount of CO absorbed from the gas phase by the blood solution in reaching equilibrium.

Theoretically it might be possible, with the more precise gasometric methods now available for measuring the per cent COHb, to increase the accuracy of the Haldane method to the degree mentioned above. In so trying, we have, however, run into certain practical difficulties, the most serious of which is the lack of constancy of  $M$  for the blood after drawing. Thus, on two occasions we found in the case of dog blood an increase of  $M$  of 50 per cent or more within 24 hours after withdrawal. The cause of such changes is obscure and is worthy of further study, for it may have a bearing on several problems in which CO has been used as a physiological reagent.

In the second type of method, the O<sub>2</sub> and CO<sub>2</sub> of the gas to be analyzed are first removed by shaking with alkaline hydrosulfite solution, and the

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

residual CO-N<sub>2</sub> mixture is then shaken with reduced blood solution, resulting in the absorption of practically the whole of the CO. In Nicloux's version of this method (3) the bound CO was estimated spectroscopically, but in subsequent modification it has been liberated by K<sub>3</sub>FeCN<sub>6</sub> and estimated gasometrically (4). In 1932 Sendroy (5) adapted this principle to the Van Slyke gasometric technique and described a method for determining 0.05 to 0.8 volume per cent of CO in air to within  $\pm 5$  per cent of the amount determined. In test analyses of known CO-air mixtures he found that only 93.7 per cent of the CO was finally recovered, so that a blank correction of 6.3 per cent had to be applied to all the determinations. In our application of the method to various physiological problems during the past 2 years we have been faced with (a) the need to improve the accuracy to within 1 to 2 per cent of the amount of CO determined, (b) the desirability of eliminating altogether the blank correction of 6.3 per cent, and (c) the extension to the lower range of 0.05 to 0.005 per cent CO in air (or less). All these aims have now been reached, our work, in fact, having been almost complete when the valuable paper of Sendroy and Fitzsimons (1) appeared. These authors here report improvements in Sendroy's original technique, as regards both ease of operation and accuracy, which now reaches the grade desired; namely, 1 to 2 per cent of the amount of CO determined. The size of the blank correction is furthermore reduced to one-third of its previous value, though the factors responsible for it have not yet been definitely established. The published method is, moreover, still limited to percentages of CO in air of 0.05 per cent and over, though the range below 0.05 per cent is now becoming of practical importance. In this paper we, therefore, describe in detail the new method devised by us for the lower range below 0.05 per cent. We have also made independent modifications of Sendroy's earlier technique (5) in the upper range and have thereby not only attained the extra precision and ease of operation recently reported by Sendroy and Fitzsimons, but have also been able to eliminate the blank correction. For the latter reason it has seemed worth while to give a somewhat more brief account of our modifications of the Sendroy method and tests in the upper range.

### *Experimental Methods and Results*

In order to measure percentages down to 0.005 to within 1 to 2 per cent of the amount of CO determined, the method must be sensitive to as little as 0.0001 per cent CO. Since the volume of CO, after isolation in the Van Slyke-Neill and Scholander-Roughton apparatus, can be measured to within about  $\pm 0.10$  cmm. with certainty, it is necessary that the volume of the gas mixture used for analysis should be not less than

150 cc. Actually tonometers of 300 to 350 cc. capacity have been used, so as to provide a margin of safety; they are of the Barcroft type but have 3-way stop-cocks at both ends instead of at only one end.

*Reagents*—As for the estimation of CO in blood (see the accompanying paper (6)) together with

(a) Fresh human blood.

(b) Borated blood solution made by mixing together 2 drops of caprylic alcohol, 1 part of the fresh human blood, 1 part of water, 1 part of 3 per cent borax ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) solution, and 2 parts of 1 per cent saponin solution. This is stored out of contact with air in a syringe. The function of the borate is partly to provide an alkaline pH and consequently a greater affinity for CO, and partly to stabilize the hemoglobin against inactivation and bacterial decomposition during the prolonged rotation of the tonometer.

(c) Krogh's hydrosulfite (modified Fieser) solution. 16 gm. of  $\text{Na}_2\text{S}_2\text{O}_4$ , 2 gm. of sodium anthraquinone- $\beta$ -sulfonate, and 14 gm. of KOH are dissolved in 100 gm. of water. This is freshly prepared each day, anomalous results having been sometimes obtained with old solutions.

*Procedure*—The tonometer is filled with 30 per cent NaCl solution, and the latter then displaced with the CO-air mixture to be analyzed, the pressure being finally brought up to  $1\frac{1}{4}$  atmospheres with the aid of a gas burette and mercury reservoir. The temperature of the gas in the tonometer is fixed by immersing it in a water bath at room temperature for a few minutes, and the gas pressure is then measured by a mercury manometer. 50 cc. of Krogh's solution are next forced from a syringe into the tonometer and the latter shaken rapidly by hand for 5 minutes to complete the absorption of the  $\text{O}_2$  and  $\text{CO}_2$ . 5 to 10 minutes later Krogh's solution is carefully run off, the pressure of the gas in the tonometer thereby becoming about equal to atmospheric; residual traces of Krogh's solution are removed by three successive washings with 5 cc. portions of distilled water. Exactly 5 cc. of the borated blood solution are then forced into the tonometer, and the latter rotated in the dark for  $1\frac{1}{2}$  to 2 hours in a water bath at room temperature to insure complete equilibration between the gas and liquid phases.

The residual CO in the gas phase, even at perfect equilibrium, is allowed for by means of the COHb dissociation curve (see below). At the end of the rotation the tonometer is stood vertically for 2 minutes to allow the blood solution to drain, and the latter is then run off into the cup of the Van Slyke-Neill apparatus and thence into the chamber. The contents of the tonometer are rinsed into the cup with 2 or 3 portions (about 3 cc. each) of distilled water to remove the blood solution quantitatively and the whole of the washings run into the Van Slyke-Neill chamber,

the volume of each washing having been read in the cup. 2 drops of caprylic alcohol and 1 cc. of borate-hydrosulfite solution are added thereto, and the analysis of the mixed solution for CO carried on exactly as described in the accompanying paper, save that (a) the amount of ferricyanide used is increased from 1.0 to 1.5 cc. and of soda for CO<sub>2</sub> absorption from 1.5 to 2.0 cc., and (b) the blood-ferricyanide solution is shaken for 10, not 3 minutes. The blank CO content of 5 cc. of the borated blood solution has been previously determined during the tonometer rotation. The percentage of CO in the air sample is calculated as in the following example: the volume of the tonometer = 305 cc.; the barometer = 749.5 mm. of Hg; the temperature of the water bath in which the tonometer was immersed = 19°; aqueous vapor pressure over 30 per cent NaCl = 16 mm. of Hg at 19°; the tonometer filled with gas mixture to positive pressure of 194 mm. of Hg; volume of the gas sample at 0° and 760 mm. of Hg equals

$$305 \times \frac{749.5 + 194 - 16}{760} \times \frac{273}{273 + 19} = 348 \text{ cc. N. T. P.}$$

The total CO content of blood solution and washings = 0.0383 cc. N. T. P.; the blank CO content of 5 cc. of blood solution used for equilibration = 0.0023 cc. N. T. P.; CO taken up by blood solution = 0.0383 - 0.0023 = 0.0360 cc. N. T. P.; per cent CO in the gas mixture less CO unabsorbed at equilibrium equals

$$100 \times \frac{0.0360}{348} = 0.0103\%$$

per cent COHb in blood solution at equilibrium in the tonometer = 19; the temperature of equilibration = 22°.

The residual per cent CO in the gas phase of the tonometer in equilibrium with 19 per cent COHb at 22° = 0.0004 (as read off from the COHb dissociation Curve A of Fig. 2 (see below)). Per cent CO in the original gas mixture = 0.0103 + 0.0004 = 0.0107 per cent.

Notes--The total CO content of the blood solution and washings in cc. N. T. P. is calculated from the expression

$$(p_{12} - p_{22}) \times \frac{l_1 - l_2}{l_1} \times \frac{f_2}{100} \times \left(1 + \frac{S\alpha}{50 - S}\right) \left(1 + \frac{3.5\alpha}{50 - 3.5}\right) \quad (2)$$

or

$$(p_{1.5} - p_{2.5}) \times \frac{l_1 - l_2}{l_1} \times \frac{f_{0.5}}{100} \times \left(1 + \frac{S\alpha}{50 - S}\right) \left(1 + \frac{3.5\alpha}{50 - 3.5}\right) \quad (3)$$

where  $p_{12}$ ,  $p_{22}$  are the respective Van Slyke readings at the 2.0 cc. mark before and after extrusion of the extracted CO from the chamber,  $p_{1.5}$ ,

$p_{2.5}$  are the similar readings at the 0.5 cc. mark,  $l_1$ ,  $l_2$  are the respective lengths of the gas samples in the capillary before and after absorption with Winkler's solution,  $f_2$  is the factor in volumes per cent of CO for the blood sample = 1 cc., and  $S$  (*i.e.* total solution extracted in Van Slyke chamber) = 3.5 cc., for measurements at the 2.0 cc. mark. The values of  $f_2$  are given in the eleventh column of Table 30 of Peters and Van Slyke (7).  $f_{0.5}$  is the similar factor for measurements at the 0.5 cc. mark, as given in the tenth column of Table 30 (7),  $S$  is the total volume of solution extracted in the Van Slyke chamber, *i.e.* 13 cc. in the present instance, and  $\alpha$  is the solubility coefficient of CO in aqueous solution at the temperature of the measurement.

It is essential that all contamination of the gas with extraneous oxygen (atmospheric or otherwise) should be avoided, especially after the absorption of  $O_2 + CO_2$  by Krogh's solution is complete. To this end, the procedure was so arranged that the gas pressure in the tonometer should be *equal* to or *greater* than the outside atmospheric pressure at every stage, thus minimizing the chance of leaks even if the tonometer taps are perfectly greased, as they must be. A check on the freedom of the gas from  $O_2$ , after absorption with Krogh's solution, is given by the behavior of the blood solution after it is introduced into the tonometer. After 2 minutes or so of shaking by hand the color of the blood solution should have changed from scarlet to the purple hue of reduced hemoglobin if the  $O_2$  has been adequately removed. (This test will, of course, be indecisive if Krogh's solution is not completely washed out before the addition of the blood solution.) Actually, with the volumes of the gas phase and blood solution used, the  $O_2$  of the latter does not quite dissociate completely even at equilibrium, but the amount remaining behind is unimportant.

The 5 cc. of blood solution used above are sufficient if the per cent CO is not above 0.03. Between 0.03 and 0.05 per cent CO, the volume should be increased to 10 cc. to insure an adequate margin of the CO-combining capacity of the blood over the CO content of the air sample. With very low CO percentages, the blank CO content of the blood solution should be as slight as possible and the blood should therefore be drawn from a non-smoker, if available.

The period of rotation of the tonometer, *viz.*  $1\frac{1}{2}$  to 2 hours, might perhaps be shortened, but this has not been tested, since a time of this order is usually consumed in the daily preparation of the Van Slyke apparatus and reagents, and in duplicate determinations of the blank CO content of the blood solutions. Actually the preparation of each individual tonometer before rotation and its subsequent analysis usually take about 1 hour, so that the average time for each gas analysis of a series of  $x$  samples



in 1 day is  $(60 + 120)/x$  minutes. Our present rotator accommodates four tonometers, so that the average time of each analysis is about 90 minutes.

Fig. 1, Curve A, shows the  $O_2$  dissociation curve of the borated human blood solution at  $25^\circ$ . The curve was readily determined by a modification of the method of Forbes and Roughton (8): 1 to 5 cc. of aerated blood solution ( $O_2$  content known by previous Van Slyke determination) were introduced into the *thoroughly cleaned* Van Slyke-Neill chamber, evacuated to the 50 cc. mark, and shaken for 15 minutes, by which time equilibrium between the gas and liquid phases was reached. The top of the chamber was then connected with a 300 cc. vacuum tonometer, and mercury slowly readmitted to the chamber by opening slightly the lower tap. The blood

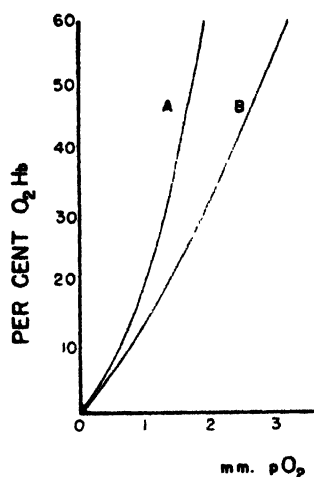


FIG. 1

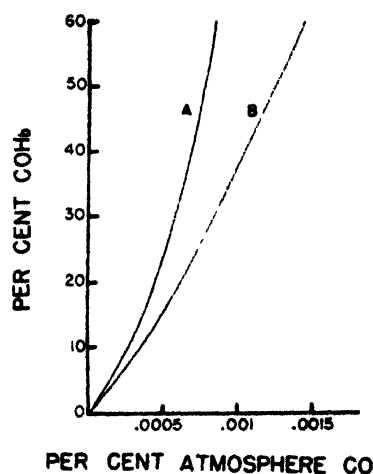


FIG. 2

FIG. 1. Oxygen dissociation curve of 1:5 blood solution at  $25^\circ$ . (Curve A, borate-saponin solution; Curve B, water-saponin solution).

FIG. 2. Carbon monoxide dissociation curve of 1:5 blood solution at  $25^\circ$ . (Curve A, borate-saponin solution; Curve B, water-saponin solution).

solution was thus driven up to the top tap of the chamber, and the extracted gas expelled, without any danger of reoxygenation of the partially reduced hemoglobin. The residual  $O_2$  content of the latter was then determined by the addition of 0.5 cc. of deaerated 30 per cent ferricyanide solution followed by the usual absorptions with alkali and hydrosulfite. From the residual  $O_2$  content, and the initial  $O_2$  content and volume of the blood solution, the  $O_2$  content and pressure of the gas phase in the Van Slyke-Neill chamber at the end of the 15 minutes shaking are readily calculated (see (8)), and the per cent  $O_2Hb$  is of course obtained by dividing the residual  $O_2$  content by the  $O_2$  capacity.

Fig. 2, Curve A, the COHb dissociation curve of the borated blood solution in the absence of  $O_2$ , is obtained from Fig. 1, Curve A, by means of the principle of Douglas, Haldane, and Haldane (9). This consists of dividing all the  $O_2$  pressures of Fig. 1, Curve A, by  $M$  (the equilibrium constant of the reaction).

$CO + O_2Hb \rightleftharpoons O_2 + COHb$  (see Equation 1). The chart so obtained gives the relation between the per cent COHb and CO pressure at equilibrium. The validity of this principle was established experimentally by Douglas and Haldane, and has recently been confirmed in a more systematic manner by Darling and Roughton (10). The value of  $M$  actually used, namely 300 at  $25^\circ$ , was obtained by separate experiments.

Curve B in Fig. 1 is the average, at  $25^\circ$ , for several workers who furnished blood from time to time for the CO analyses. Appreciable variations from the mean were noted in certain individuals but these did not exceed 1 in 10, which would have an insignificant effect on the calculation of the unabsorbed CO, since the latter as a rule did not exceed 4 per cent of the total CO. At  $20^\circ$  the CO pressures of Curve B, Fig. 1, need to be multiplied by a factor of 0.6 and at  $30^\circ$  by 1.5. At intermediate temperatures the required factor can be arrived at accurately enough by numerical interpolation.

*Tests and Accuracy of the Method*--Table I shows the results of a series of tests in which the per cent CO was determined in CO-air mixtures prepared in two different ways. In Method A the tonometers were first filled to a pressure of  $1\frac{1}{4}$  atmospheres with air containing less than 0.0001 per cent CO. The pressure, temperature, and volume of the tonometers having been measured, their dry air contents in cc. at  $0^\circ$  and 760 mm. of Hg were readily calculated. Known volumes of CO of analyzed purity were then measured in the chamber of the Van Slyke manometric apparatus (at the 2.0 and 0.5 cc. marks) and forced from there into the tonometers. For the CO percentages above 0.01, carbon monoxide gas prepared from formic and sulfuric acids was used, the  $CO_2$  and  $O_2$  having been removed by previous shaking with Krogh's solution. For the lower CO percentages, the chemically prepared carbon monoxide was first diluted to a known extent with air before measurement in, and transfer from, the Van Slyke apparatus.

In Method B the tonometers were filled with CO-free air as before, but the measured amounts of CO were then introduced, not in the form of gas but in solution in water. The CO solution was prepared as follows: A 300 cc. tonometer was filled half with water and half with CO gas, and then shaken for some minutes until equilibrium between gas and liquid was reached. The CO gas was then expelled by connecting the tonometer at its lower end with a mercury reservoir. The top end of the tonom-

eter was connected to a calibrated burette, graduated in hundredths of a cc., with a mercury leveling bulb also attached, all air bubbles having been carefully flushed out from the joints by running through excess of the solution. The whole system was kept under positive pressure to prevent formation of small gas bubbles, and the CO content of the solution thus remained constant for long periods. Its value, in volumes per cent CO to within 1 in 200 of the amount present, was determined by transferring 5 cc. portions anaerobically to the Van Slyke manometric apparatus and then liberating and measuring the CO by a simple modification of

TABLE I

*Comparison of Observed and Expected Percentages of Carbon Monoxide in Air Mixtures over Range 0.001 to 0.06 Per Cent CO*

	Observed CO	Expected CO	Recovery
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Method A, mixture prepared from CO gas	0 0013	0.0013	
	0 0042	0.0043	
	0 0091	0.0093	98.0
	0.0135	0.0136	99.3
	0 0213	0.0214	99.5
	0.0253	0.0256	98.8
	0 0295	0 0301	98.0
Mean.....			98 7
Method B, mixture prepared from CO solution	0.0012	0.0011	
	0 0022	0.0022	
	0.0036	0.0035	
	0.0058	0.0058	
	0.0119	0.0117	101 6
	0 0120	0.0119 <sub>5</sub>	100.4
	0.0179	0.0175	102 3
	0 0278	0.0274	101.4
	0.0418	0.0425	98.4
Mean.			100 8

our procedure for estimating the CO content of blood (6). Measured volumes of the CO solution were similarly transferred anaerobically from the burette to the tonometers, which were then shaken for 2 to 3 minutes so as to liberate the whole of the CO into their gas phases. With a tonometer containing 300 cc. of air (N. T. P.) and 1 cc. of CO solution (content, say, 2.0 volumes per cent) the expected per cent CO would thus be 0.02/3 or 0.0067 per cent CO.

We believe that the uncertainty in the CO content of the air mixtures was only 1 part (or less) in 100 of the amount present, whichever method was used, but we are inclined to place greater reliance on the results obtained with the CO solution technique, since this was easier to run, once the stock of CO-water had been prepared and its content estimated.

The outside air of New York sometimes contains a few parts per million of CO,<sup>1</sup> so that this was used only as the source of CO-free air when analysis by the present method indicated a CO percentage of 0.0001 or less (*i.e.*, no CO within the limits of experimental error).

Inspection of the figures given in Table I shows that in the very low range (*i.e.* below 0.009 per cent CO) the observed and expected results agree to within 0.0001 per cent CO, which is about the limit of sensitivity of the method. Above 0.009 per cent CO, the percentage recovery is seen to range from 98 to 102.3, the average value for mixtures by Method A being 98.7 per cent and by Method B, 100.8 per cent. In view of the possible uncertainty of  $\pm 1$  part in 100 in the expected CO figures, it seems that these results indicate practically complete recovery within experimental error.

The method was further checked by means of two CO-air mixtures kindly furnished to us by Dr. Martin Shepherd of the Department of Gas Chemistry, Bureau of Standards. With the first mixture we obtained the figures 0.0107, 0.0108, 0.0109, 0.0109, mean  $0.0108 \pm 0.0001$  per cent CO. The expected figure, based on the method of mixtures by which the sample was prepared, was also  $0.0108 \pm 0.0001$ , but was not made known to us until after we had completed our analyses. With the second mixture our figures were 0.0393, 0.0395, 0.0389, mean  $0.0392 \pm 0.0004$  per cent, as against an average figure of 0.0393 per cent CO obtained by analyses of the same mixture in other laboratories by methods not involving the use of blood as the active reagent.

#### *Upper Range of 0.04 to 0.7 Per Cent CO in Air*

*Procedure*—A modified Hempel pipette ((7) Fig. 11, p. 109) is filled with Krogh's hydrosulfite solution, the cup, the bore of the 3-way tap, and the side tube being filled with water. The side tube is then connected by rubber tubing with the gas to be analyzed, and sufficient of the latter passed through the side tube, tap, and cup of the Hempel pipette to flush out all traces of air. The 3-way tap is then turned to connect the gas with Krogh's solution, and the Hempel bulb filled with the gas, suction if necessary being applied to the other bulb of the pipette. The tap is then sealed with mercury and the O<sub>2</sub> and CO<sub>2</sub> of the gas absorbed by

<sup>1</sup> The amount varies with the weather, traffic, and industrial conditions.

gently shaking the Hempel pipette for 5 minutes and then allowing it to stand for 5 to 10 minutes more.

During this time the Van Slyke-Neill chamber is prepared in the following way. It is thoroughly cleaned after the previous analysis; then 5 cc. of water are evacuated and shaken in the chamber for  $1\frac{1}{2}$  minutes, after which the mercury reservoir is lowered so as to draw the evacuated water well down into the rubber connection between the chamber and the rest of the Van Slyke apparatus, in this way disengaging bubbles frequently trapped there. The evacuated water and extracted air are then completely expelled, and 4 drops of caprylic alcohol, followed by 1 or 2 cc.<sup>2</sup> of fresh human blood (preferably from a non-smoker), 2 cc. of 1 per cent saponin, and enough water to make a total volume of 5 cc. are drawn into the chamber. The tap is sealed, the mercury lowered to the 50 cc. mark, and the chamber covered with tin-foil or black paper to prevent dissociation by light of any COHb present in the blood during the 4 minute shaking which follows. The extracted gases are quantitatively expelled without loss of blood solution, the tap resealed, and the reading of the manometer taken with the mercury solution meniscus at the 50 cc. mark ( $p_1$ , temperature  $t_1^\circ$ ). The contents of the chamber are then adjusted to atmospheric pressure (or just below) by admitting mercury from the reservoir and 10 to 40 cc. of gas admitted to the chamber from the Hempel pipette, according to the procedure illustrated in Fig. 11 of Peters and Van Slyke's text-book (7). The volume of gas chosen depends on the approximate percentage of CO in the gas mixture, a smaller volume being taken if the percentage is high. The tap of the Van Slyke chamber is sealed, the mercury-liquid meniscus is again set at the 50 cc. mark, and the manometer read =  $p_2$ .

The volume of gas drawn into the chamber then =  $V$  =

$$45 \times \frac{273}{273 + t} \times \frac{p_2 - p_1}{760} \text{ cc. N. T. P.} \quad (4)$$

The figure 45 represents the difference between the volume of the chamber (50 cc.) and the volume of the blood solution (5 cc.).

If the air to be analyzed originally contained  $x$  per cent  $O_2$  and  $y$  per cent  $CO_2$ , its volume, after absorption in the Hempel pipette, will be reduced in the ratio  $100 - (x + y)$  to 100.

In the case of ordinary air  $x + y = 20.96$  or 21, so that the original volume of air used for analysis =  $V/0.79 = 1.26V$  cc. N. T. P.

In other cases an exactly measured volume of the gas to be analyzed (not more than 40 cc.) must be forced into the Hempel pipette at the

<sup>2</sup> 2 cc. are used if the per cent CO in the gas is greater than 0.25.

outset, and the whole residue of the gas after deoxygenation is transferred from the Hempel pipette to the Van Slyke-Neill chamber. The original volume of gas sample is reduced to N. T. P. in the usual way.

After the measurement of gas into the chamber is completed, the latter is covered with dark paper and gently shaken (with the mercury at the 50 cc. mark) for 30 minutes (as in Sendroy's technique), at the end of which the equilibrium between the hemoglobin and the gas phase is complete (assuming that the room temperature is not less than 20°). The gas is then quantitatively expelled from the chamber without any loss of blood solution, and 2 cc. of the usual  $\text{Na}_2\text{S}_2\text{O}_4$ -borate solution drawn in from the cup. From this point onwards the remainder of the procedure is the same as that described in our accompanying paper on estimation of blood CO content. The per cent CO in the gas mixture is then calculated in just the same way as in the example given in the procedure for the lower range of CO percentages, allowance again being made, with the aid of the dissociation Curve B of Figs. 1 and 2, for the CO still remaining in the gas phase even at equilibrium. The effect of this last factor is, however, much less important than in the lower range, for the correction due to it amounts only to 1 part in 50 even at the bottom of the present range, where the effect is most marked. Sendroy and Fitzsimons used 5 times as much blood as we customarily do, so that in their case this factor would be nearly 5 times less important than in ours. Only, indeed, in the most dilute CO mixtures analyzed by them would we expect it to be responsible for any appreciable part of their failure to obtain complete recovery.

*Tests and Accuracy of the Method*—Accurately measured volumes of air and of CO, of analyzed purity, were forced into the Hempel pipette, mixed there, and deoxygenated, and aliquot samples then returned to the Van Slyke-Neill chamber for analysis as described above. Table II summarizes the results of our sixteen most recent tests with the technique at its best.

The expected percentage, as calculated from the volumes of air and CO mixed together, is subject to a possible error of  $\pm 1$  per cent of the figures given in this column.

It will be seen that the per cent recovery ranges from 97.5 to 101.5; i.e., over a span of four, and that all but four of the per cent recoveries fall within the range 98.5 to 100.0. The average per cent recovery in Table II, viz. 99.5, may well be considered, within experimental error, to be complete. In view of the 1 per cent uncertainty in the expected figures we feel justified in concluding that the experimental error of the method is proved to be no more than 1 to 1.5 per cent of the amount of CO determined, over the range 0.03 to 1.00 per cent CO in air. The final limit of sensitivity of the method we believe to be about 0.0005 per

cent. The figure is based on the volume of gas sample used (30 to 40 cc.) and the minimum amount of CO (*viz.* 0.10 to 0.15 c.mm.) measurable with certainty by the combined Van Slyke-syringe technique.

A further check of the method was provided by eight measurements on the 0.0393 per cent CO in air mixture supplied by the Bureau of Standards (see above). The figures obtained by us were 0.0405, 0.0392, 0.0395, 0.0392, 0.0385, 0.0395, 0.0394, 0.0386, mean = 0.0393 per cent CO. Aside from the 0.0405 reading, which is somewhat off line, all the other figures lie within the range  $0.0391 \pm 0.0005$  per cent. Such agreement with the expected figure is additional evidence of the completeness of recovery.

TABLE II

*Comparison of Observed and Expected Percentages of Carbon Monoxide in Air Mixtures over Range 0.03 to 1.00 Per Cent CO*

Observed per cent	Expected per cent	Per cent recovery
0.6783	0.674	100.5
0.6597	0.674	98.0
0.6658	0.674	98.8
0.4596	0.452	101.5
0.4536	0.452	100.3
0.4576	0.452	101.2
0.4525	0.452	100.1
0.3451	0.346	99.7
0.3412	0.346	98.5
0.2876	0.286	100.5
0.1296	0.133	97.5
0.0736	0.0742	99.2
0.0708	0.0712	99.4
0.0689	0.0697	98.9
0.0649	0.0657	98.8
0.0472	0.0474	99.6
Average. ....	...	99.5

## DISCUSSION

The method for the lower range of CO percentages (below 0.03) is admittedly a laborious one and requires meticulous attention to detail at every stage. No single one of the numerous operations involved should, however, present difficulty to a person well trained in the standard methods of blood gas analysis. As there are many such workers in different laboratories, the technique might therefore be of fairly wide use, not only for precise studies such as those relating to the mode of CO elimination from the human body (to be published later), but also as a reference method for testing less accurate but more expeditious methods of estimating CO in air.

Our modified method for the upper range (0.03 to 1.0 per cent CO in air) differs from that of Sendroy in several particulars, of which the most noticeable are (a) the removal of  $O_2$  and  $CO_2$  from the gas mixture before introduction into the Van Slyke apparatus (the reason for this is mainly one of convenience); (b) the absorption of the CO by fresh saponin-laked human blood solution (1:5 instead of 1:2); (c) the quantitative correction, by means of the dissociation curve, for the residual CO in the gas phase at equilibrium; (d) the greater accuracy of analysis of the isolated CO, with the aid of the Scholander-Roughton technique. One or more of these changes, with the exception of (c), is presumably responsible for our recoveries being practically 100 per cent instead of 98 per cent complete, as in Sendroy's recent method. Though we have not analyzed the matter in detail, we are inclined to believe that factor (b) is probably the most important. The presence of the saponin and the greater dilution of the blood (1:5, as compared with 1:2 in Sendroy's method) insure the absence of clumps of stromata, which might entangle hemoglobin and protect it from the subsequent action of the ferrieyanide. The saponin may also protect the hemoglobin from inactivation at the mercury-solution or gas-solution interfaces during the shaking. The borate, in the lower range method, almost certainly exerts a stabilizing influence and would have been used also in the upper range method, had this been thought of in time.

Sendroy leaves it open as to whether the great improvement in his early recovery figures of 93.7 (5) to 98 per cent (1) with more dilute laked blood is due to the greater speed of combination with CO or to the greater affinity for CO. According to Hill and Wolverkamp (11) the affinity of hemoglobin in the red cell for  $O_2$  or CO is less than when it is in solution, but even so the residual pressure of CO in the gas phase at equilibrium should have been less than 1 per cent of the original CO pressure under the conditions of Sendroy's experiments (5). On the other hand, hemoglobin solutions when shaken with gas do undoubtedly take up CO far more rapidly than do red cell suspensions, owing to the far more intimate contact, in the former case, between the hemoglobin molecules and CO molecules at the gas-liquid interface. Other detailed examples of this principle are given by Roughton (12) in his paper on the influence of diffusion in manometric measurements of certain rapid biochemical reactions; it is on this account, indeed, that we have from the outset made use of hemoglobin solutions rather than of whole blood for our CO absorption work.

Our measurements have so far been limited to carbon monoxide in normal inspired and expired air. The reader is referred to the paper of Sendroy and Fitzsimons (1) for the special precautions necessary in dealing with industrial gases containing large amounts of hydrocarbons.



## SUMMARY

Sendroy's methods of analyzing small amounts of CO in air, with blood as the active reagent, have been modified and extended. With gas samples of 400 to 500 cc., combination of the Van Slyke and Scholander-Roughton techniques makes it possible to analyze CO percentages in the range 0.03 to 0.005 to within 1 to 1.5 per cent of the amount present. The maximum sensitivity of the method is about  $\pm 0.0001$  per cent CO. In the higher range (0.03 to 1.0 per cent) only 40 to 50 cc. gas samples are required; the accuracy is again 1 to 1.5 per cent of the amount present, but the maximum sensitivity is about  $\pm 0.0005$  per cent CO. In both ranges the recoveries obtained with test mixtures were 99 to 100 per cent; *i.e.*, complete within the limits of experimental error.

Our thanks are due to Dr. Julius Sendroy, Jr., for various critical suggestions.

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## PECTIC ENZYMES

### VI. THE USE OF AN ION EXCHANGE RESIN FOR THE COMPLETE REMOVAL OF PECTIN-METHYLESTERASE FROM COMMERCIAL PECTINASES\*

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Two enzymes which act on soluble pectic substances are recognized at the present time; namely, pectin-polygalacturonase (PG), which catalyzes the hydrolysis of the 1-4 glycosidic linkages of polygalacturonic acids, and pectin-methylesterase (PM), which catalyzes the hydrolysis of the methyl ester groups. The latter enzyme, PM, occurs in nature practically free of PG (9) and has been employed for the deesterification of pectinic acids (5). On the other hand PG appears always to be accompanied by PM (7). Consequently, all reports dealing with the enzymatic decomposition of the polygalacturonic acid components of pectinic acids are concerned with cases in which this reaction was accompanied by demethylation.

For some time we have been desirous of obtaining some PG entirely free of PM activity. In 1938 a study of the separation of these two enzymes was undertaken in this laboratory<sup>1</sup> without reaching the goal of complete separation. During this work, it was observed that PM shows considerable sensitivity towards acidities below pH 3, while PG withstood prolonged exposure to such conditions. Table I shows the results of a typical experiment. This difference in behavior was further accentuated when the solutions were heated at low pH values. Partial destruction of PM (90 per cent) was accomplished by such methods, but no PG entirely free of PM was obtained. Rothschild (10) in 1939 reported some work done on similar lines, but used methods of determining activities which had been previously shown by Kertesz (8) to be unreliable. The development of ion exchange resins and their increased application in biological work, especially for the separation and determination of amino acids (1, 2), suggested their employment for the separation of PM and PG. The different behavior of these two enzymes towards acids, and the fact that they have different pH optima, made it likely that they would behave differently on ion exchange columns.

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<sup>1</sup> Moyer, J. C., and Kertesz, Z. I., unpublished results.

The present report deals with the removal of PM from two commercial pectinases by the use of ion exchange resins. Since we believe that this method will prove applicable to the separation of some other enzymes, the difficulties encountered during this work are also discussed below.

#### EXPERIMENTAL

Two commercial enzyme preparations, designated as Pectinase I (Pectinase 8)<sup>2</sup> and Pectinase II (Pectinol 46AP), were used in this work without any further purification. Most of the experiments were conducted with Amberlite IR-100 operating in acid cycle in a closed system column 105 cm. long, 2.5 cm. in diameter, and holding 340 gm. of resin at close packing. The column was fitted with a 2-hole rubber stopper at the top, through which connections were made to a vacuum pump and to a funnel which was clamped beside the column near the top. After

TABLE I

*Effect of pH during Holding for 2 Hours at 25° on Activity of Pectin-methylesterase (PM) and Pectin-polygalacturonase (PG) in Commercial Pectinase*

pH	Activity retained	
	PG	PM
	<i>per cent</i>	<i>per cent</i>
3.0	100	92
2.4	100	78
2.0	100	53
1.6	90	49
1.0	40	12

the resin had been initially packed by backwashing, it was prepared for each experiment in the following manner: The resin was washed with about 1 liter of distilled water by the downflow at 4 to 6 cc. per minute, then by 35 per cent HCl at the same rate, followed by another water wash until no Cl<sup>-</sup> could be detected in the effluent. At this point the addition of water to the column was stopped and the liquid left in the column drained out until the top 3 cm. of resin were free of water. The enzyme solution to be treated (about 200 cc.) was placed in the funnel and introduced by vacuum through the top of the column with enough force to penetrate the drained layer of resin, and the column filled to 15 cm. above the resin layer. The vacuum line was closed and the downflow was then begun by opening the stop-cock at the bottom of the column.

<sup>2</sup> We are indebted to the Rohm and Haas Company, of Philadelphia, Pennsylvania, for these enzyme preparations, and to the Resinous Products and Chemical Company, of Philadelphia, Pennsylvania, for various samples of ion exchange resins.

The rate was regulated to 4 to 6 cc. per minute. The flow of additional enzyme solution from the funnel to the column was maintained by the vacuum created in the column by the outflow. The enzyme solution was followed by water introduced in the same manner.

PM was determined by the titration method (8) at pH 6.2. The rapid approximate method of Fellers and Rice (4) for the estimation of pectic acid was adapted to the estimation of PG activity in preference to the more accurate but time-consuming calcium pectate method (3). The reaction mixture contained 10 cc. of approximately 1 per cent pectinic acid solution, 0.1 cc. of 10 per cent gelatin, and amounts of the enzyme solution varying from 0.5 to 2.0 cc. The pH was adjusted to 3.5 and the volume made up to 12 cc. with water. Gelatin was used to protect the PG, especially at low concentrations. The changes which occurred in the pectic acid content of this mixture at 30.0° were followed. Under these conditions, the log of the volume of pectic acid precipitate plotted against reaction time gave a straight line, until at least 60 per cent of the precipitate had disappeared. The time in minutes ( $t_h$ ) required for the enzyme to reduce the volume of the precipitate to half of its initial value was graphically interpolated and the relative PG activity expressed by the following arbitrary formula

$$\text{PG units} = \frac{100}{(t_h)(\text{gm. pectinase})}$$

The buffered pH values were obtained by adding potassium acid phthalate or sodium dihydrogen phosphate and raising or lowering the pH to the desired value with dilute NaOH or HCl. Unless otherwise stated, the data on treated enzymes in Table II are based on aliquots taken from the mixed, complete effluents. Dry matter determinations were made for the calculation of PG activities per gm. of dry matter. The results obtained are shown in Table II.

*Preparation of Solid PM-Free Pectinase*—Two dry preparations were obtained from a 2 per cent solution of Pectinase I which had been shaken for 5 minutes with each of three batches of 30 gm. of resin and passed through a 50 cc. burette (containing 30 gm. of acid cycle resin) at the rate of 4 cc. per minute. The first preparation, A, was obtained from this effluent by precipitating with 10 volumes of 95 per cent ethanol and the second preparation, B, was obtained from the filtrate of the above by the addition of 5 volumes of dry ether. A dry preparation, C, was obtained from the effluent of a 2 per cent solution of Pectinase II (treated on the column), by the addition of 2 volumes of alcohol (containing a trace of  $\text{CaCl}_2$ ) and 2 volumes of ether. A fourth preparation, D, was obtained in the same manner from the effluent of a 4 per cent solution of Pectinase

II treated on the column. The precipitates were washed with dry ether and dried *in vacuo* over  $\text{CaCl}_2$ . The yields of the dry preparations, expressed as the percentage of the weight of dry pectinase which was

TABLE II

*Removal of Pectin-methylesterase (PM) by Amberlite IR-100, and Recovery of Pectin-polygalacturonase (PG) from Commercial Pectinases*

Experiment No.	Pectinase No *	Treatment	pH		Dry matter		Activities in effluents and preparations per gm. dry matter, as per cent of original	
			Before adsorption	After adsorption	Before adsorption	After adsorption	PG	PM
					per cent	per cent		
1	I	None	6.1		4.0	3.0	50	0
2	"	In 0.5% $\text{NaH}_2\text{PO}_4$	8.1	4.0	4.3	2.8	65	3
3	"	" 0.5% "	7.0	3.5	4.3	2.5	70	2
4	"	" 0.5% $\text{KH}_2\text{C}_2\text{O}_4$	6.1		4.2	2.2	60	0
5	"	" 0.5% "	4.1		4.2	2.6	20	0
6	"	" 0.7% $\text{KCl}$	2.4	1.5	4.1	2.6	0	0
7	"	Preparation A	6.1	3.0	2.0		150	0
8	"	" B	6.1	3.0	2.0		125	0
9	II	None	6.3		3.9	2.7	45	47
10	"	In 0.5% $\text{KH}_2\text{C}_2\text{O}_4$	6.1	2.5	2.2	0.9	90	51
11	"	" 0.5% "	4.9	2.0	2.2	1.0	80	30
12	"	" 0.5% "	3.9	1.5	2.2	1.0	80	11
13	"	" 1.0% "	3.9		2.2	1.0	80	0
14	"	" 1.0% "	3.4	1.5	2.2	1.0	90	0
15	"	" 1.0% "	2.9	1.2	2.5	1.0	90	0
16	"	As in (15). First 10 cc. collected in 2.5 min.	2.9		4.3	1.0	220	0
17	"	Subsequent 250 cc. collected from (16) in 1 hr.	2.9		4.3	1.4	75	0
18	"	In 1.0% $\text{KH}_2\text{C}_2\text{O}_4$	2.3	1.0	2.5	1.5	50	0
19	"	" 1.0% "	1.8	1.0	2.7	1.6	50	0
20	"	" 0.7% $\text{KCl}$	1.8		2.7	1.7	45	0
21	"	Preparation C	2.9	1.0	2.0		20	0
22	"	" D	2.9	1.0	4.0		20	0

\* Initial activities in Pectinase I were 77 PM units and 240 PG units per gm.; in Pectinase II, 109 PM units and 565 PG units per gm.

treated, were 43, 15, 6, and 33 per cent for Preparations A, B, C, and D, respectively. The emphasis in this work being on the qualitative aspects of the removal of PM, no attempts were made at this time to recover a maximum proportion of the PG. The activities of the dry preparations are shown in Table II.

## DISCUSSION

Complete removal of PM from Pectinase I was accomplished at pH 6.1 or below, from Pectinase II (in the presence of a suitable buffer) at pH 3.9 or below. The results given in Table I indicate that the removal of PM did not occur simply by action of high acidities. The possibility cannot be overlooked, however, that specific conditions characteristic to the column may cause inactivation of the PM without its actual removal from the solution. If the PM is removed through ion exchange, it is reasonable to assume that the enzymes would not be efficiently adsorbed on a cation exchanger at pH values above their isoelectric point. As the pH is lowered below the isoelectric point, the enzymes become ionized as cations and thus will be removed from the solution. The removal of PM but not of PG will be accomplished where the pH of the solution is below the isoelectric point of PM but still above that of PG.

Table II shows that different buffer concentrations were needed, in addition to different pH values, in order to effect removal of PM from Pectinase II. Titration curves for the two enzymes showed the presence of a large buffering capacity between pH 7 and 8 in the case of Pectinase II, whereas Pectinase I gave the regular S-curve. The presence of these buffers in Pectinase II may have affected the ionization of the enzymes as ampholytes, or may have actually competed with them for the exchange reaction. The effect in both cases would be to lower the pH values needed for the separation.

The high activities of dry Preparations A and B might be attributed to the removal of inert materials both in the column and during precipitation. The low activities found in Preparations C and D are undoubtedly due to the greater acidity of the effluent (pH 1) and to difficulties in precipitation and filtration which extended the length of operation to several hours. As shown by Table I, the PG activity decreases while these solutions are held at high acidities. No doubt higher yields of PG could be obtained by improving the technique and speed of operation.

The absence of active PM, when complete removal is indicated in Table II, was confirmed by methyl ester determinations (6) on the reaction mixtures after 24 hours at pH 6. These showed that no deesterification of the pectinic acid occurred.

## SUMMARY

1. Pectin-methylesterase (PM) was removed from two commercial pectinase preparations by the action of a cation exchange resin and varying proportions of pectin-polygalacturonase (PG) were recovered.

2. Pectin-methylesterase (PM) was removed from Pectinase I simply by the action of the resin on water solutions of the enzyme. It was

necessary to use buffered solutions at a low pH to accomplish the removal of PM from Pectinase II.

3. The possible mechanism of the removal and the cause of the difference in the behavior of the two pectinases are discussed.

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# THE FATE OF TRYPTOPHANE IN PYRIDOXINE-DEFICIENT AND NORMAL DOGS\*

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It has been shown that in the pyridoxine-deficient rat tryptophane is converted to xanthurenic acid (1). Xanthurenic acid was first identified by Musajo (2) in the urine of rats and rabbits fed a high protein diet, but was not found in the urine of similarly fed dogs. On pyridoxine-deficient diets, dogs have been shown to excrete a green pigment-producing compound, presumably xanthurenic acid, in the urine (3), but in minute amounts compared with that excreted by the rat. Xanthurenic acid has been found in the urine of pyridoxine-deficient swine (4), and the amount excreted was increased when tryptophane was administered (5, 6). It seemed worth while to determine the fate of tryptophane in pyridoxine-deficient dogs.

Young cocker spaniels reared in the laboratory colony were maintained from the time they weighed 3 kilos, at 6 to 8 weeks of age, on purified basal diets of the percentage composition: vitamin-free casein 45.8 or 18.0, corn-starch 20.3, sucrose 20.0 or 47.8, hydrogenated cottonseed oil 10.0, Salt Mixture 5<sup>1</sup> 2.4, and calcium carbonate 1.5. Part of the carbohydrate was supplied as corn-starch because of our difficulty of obtaining normal growth in young dogs on a purified diet when sucrose alone is used as the source of carbohydrate. This basal diet was supplemented with the crystalline vitamins at the following levels, in mg. per kilo per day: thiamine and riboflavin 0.1, nicotinic acid amide 2.0, calcium pantothenate and *p*-aminobenzoic acid 3.0, choline 5.0, inositol 25.0, fullers' earth-treated rice bran concentrate 500. In addition, fish liver oil sufficient to provide 800 I. U. of vitamin A and 70 I. U. of vitamin D per kilo and 1 gm. of wheat germ oil were given daily.

The rice bran extract, which was fed to provide biotin and the unknown factors, was prepared as follows: 5 liters of the concentrate<sup>2</sup> diluted with an equal volume of water were shaken for  $\frac{1}{2}$  hour with 1500 gm. of fullers'

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<sup>1</sup> Salt Mixture 5 of the following composition:  $\text{KH}_2\text{PO}_4$  20.3,  $\text{KI}$  0.005,  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  56.3,  $\text{NaF}$  0.068,  $\text{MgSO}_4$  4.9,  $\text{MnSO}_4$  0.021,  $\text{NaCl}$  22.4,  $\text{KAl}(\text{SO}_4)_2$  0.006,  $\text{Fe}$  citrate 2.2,  $\text{Ca}$  lactate 98.7.

<sup>2</sup> The rice bran extract was that marketed as Galen "B" by the Galen Company, Inc., Berkeley, California.



earth, filtered, and washed with 3 liters of water. This process was repeated four times and the combined filtrates were concentrated to 10 liters. The resulting extract was free of pyridoxine, as shown by rat assay. The untreated rice bran concentrate was fed to the control animals in similar amount. In addition, the control animals received 0.1 mg. of pyridoxine per kilo per day. Other members of these litters were similarly fed but deprived of other B vitamins, in order to produce parallel single deficiencies in comparable animals.

Hemoglobin determinations (7) were made at intervals to determine the severity of the pyridoxine deficiency. The presence of xanthurenic acid in the urine was qualitatively determined with ferric ammonium sulfate (8), kynurenic acid by acidifying the urine with sulfuric acid (9), and kynurenine by the method described by Kikkawa (10).

For these experiments, thirteen pyridoxine-deficient and ten normal dogs from several closely related litters were used. Four deficient and two normal dogs received the 18 per cent casein diet, all others the 45.8 per cent casein diet.

Early in the study it was discovered that both xanthurenic and kynurenic acids were unstable in dog urines even at refrigerator temperatures. To detect the presence of these substances it was necessary, therefore, to examine the urine samples immediately after they were voided.

The normal dogs excreted kynurenic acid usually for 2 to 9 hours after the feeding of tryptophane, but the deficient dogs often excreted xanthurenic acid for 24 hours, and generally the more severe the deficiency the longer the period of such excretion.

There was no excretion of xanthurenic acid by the pyridoxine-deficient dogs on either the 18 or the 45 per cent casein diet, except after the tryptophane was given. This differs from the experience of Lepkovsky and Nielsen (8) with rats, Musajo (2) with rats and rabbits, and Cartwright *et al.* (6) with swine, who noted xanthurenic acid production on high protein or pyridoxine-deficient diets without the addition of tryptophane.

*Metabolism in Prolonged Deficiency*—The results of the administration of 2 gm. of *dl*-tryptophane<sup>3</sup> or of 1.5 gm. of *l*(-)-tryptophane to nine dogs which had been maintained on the high protein basal diet for relatively long periods are shown in Table I. Dog 443 was given the amino acid six times, first after 126 days on the pyridoxine-deficient diet, and again after varying periods up to 407 days. 5 days after the first test, the animal collapsed and appeared about to die of the deficiency, the symptoms of which had apparently been aggravated by the feeding of tryptophane. At this time 1.7 mg. of pyridoxine hydrochloride were given by mouth.

<sup>3</sup> Part of the tryptophane was supplied by Merck and Company, Inc., Rahway, New Jersey.

TABLE I

*Urinary End-Products of Tryptophane Metabolism in Normal and Pyridoxine-Deficient Dogs Which Received Basal Diet Containing 45.8 Per Cent Casein*

Dog No.	Diet	Period on diet days	Hemoglobin gm. per 100 gm. blood	Amino acid fed	Period after tryptophane feeding hrs.	Urinary end-products			Comment
						Xanthu- renic acid	Kynu- renine	Kynu- renic acid	
443 ♂	Pyridox- ine-defi- cient	126	7.7	2.0 gm. <i>dl</i> -tryptophane	3	++	++	-	Complete col- lapse and pa- ralysis; 5 days after tryptophane was fed, 1.7 mg. pyridox- ine given, with immedi- ate improve- ment
					4	+++	+++	-	
					5	++	++	-	
					7	++	-	-	
		189	9.1	" "	4	+++	+	-	Vomited at in- tervals
					10	++	+	-	
		217	10.6	1.5 gm. <i>l</i> (-)-tryptophane	3-19	+++	+	-	No symptoms
					20	-	-	-	
		278	9.3	" "	3-10	+	+	-	" "
					12	-	-	-	
		311	12.8						Maximum Hb level ob- tained
		407	5.2	1.5 gm. <i>l</i> (-)-tryptophane	5	+	+	-	
					8-23	+++	+	-	No symptoms
					24	-	-	-	
445 ♂	Normal	126	13.9	2.0 gm. <i>dl</i> -tryptophane	3	Trace	+	-	" "
					5	"	+	+++	
					7	-	-	-	
		189	16.2	" "	4	+	+	+++++	" "
					6	-	+	+++	
					10	-	-	-	
		217	17.0	1.5 gm. <i>l</i> (-)-tryptophane	3-9	-	+	+++++	" "
					10	-	-	-	

TABLE I—*Concluded*

Dog No.	Diet	Period on diet days	Hemoglobin <i>gm per 100 gm. blood</i>	Amino acid fed	Period after tryptophane feeding hrs.	Urinary end-products			Comment
						Xanthu- renic acid	Kynu- renine	Kynu- renic acid	
441 ♂	Nicotinic acid-deficient; lacking unknowns	278	16.3	1.5 gm. l(-)-tryptophane	2-5 6	- -	+ -	++++ -	No symptoms
440 ♀	Normal	189	16.0	2.0 gm. dl-tryptophane	4 6 10	Trace " -	+ + +	+ +++ -	" "
451 ♂	"	181	17.7	1.5 gm. l(-)-tryptophane	3-9 10	- -	+ -	++++ -	" "
452 ♀	Pyridoxine-deficient	278	18.9	" "	3-6 7	- -	+ -	+++ -	" "
		181	9.4	" "	3-19 20	+++ -	+ -	- -	Partially collapsed; refused food for 10 hrs.
		278	8.0	" "	2-4 7	++ ++++	+ +	- -	Vomited at intervals; no collapse
		188	5.8	" "	4 8	++ +++	+ +	- -	Vomited at intervals
		188	8.5	" "	5-7 11	++ -	+ -	- -	" "
462 ♂	Normal	209	16.9	" "	3-5 6	- -	+ -	+++ -	No symptoms

Dog 443 improved in activity, appetite, and hemoglobin content of the blood at once. No more pyridoxine was given, but the hemoglobin rose gradually from 7.7 to 12.8 per cent in 6 months. 3 months later the hemoglobin level had fallen to 5.2. The later tryptophane feedings produced much less vomiting, failure of appetite, and collapse than did the first two experiments. In all six experiments on Dog 443, large amounts of xanthurenic acid were excreted in the urine following the feeding of tryptophane, and kynurenine was excreted also, but no kynurenic acid in

any case. Thus, 58 days after the curative dose of pyridoxine had been given, the metabolism of tryptophane was abnormal in character.

*Effect of dl-Tryptophane*—The normally fed dog, No. 445, examined similarly after 126, 189, and 217 days on the diet, excreted kynurenic acid and kynurenine but no xanthurenic acid when *l*(-)-tryptophane was given. When the *dl* form was given, however, a trace of xanthurenic acid was excreted during the first 3 or 4 hours. This was noted also in the case of the normal dog, No. 440. In no other case was xanthurenic acid excreted by pyridoxine-fed dogs. It is possible that *d*-tryptophane may to some extent follow an abnormal course of metabolism even in normal animals.

The other three pyridoxine-deficient dogs, which were given *l*(-)-tryptophane after 181 to 278 days on the diet, also excreted only kynurenine and xanthurenic acid but no kynurenic acid. All of these animals vomited at intervals after the tryptophane was administered.

*Metabolism in Early Deficiency States*—In Table II are given the results of similar studies with young dogs after 30, 65, 92, and 100 days on the basal diets containing 18.0 or 45.8 per cent casein. After 30 days, when the dogs were about 3 months old, the normal animal on high protein diet had 13.9 per cent hemoglobin and the pyridoxine-deficient dogs 10.7 and 10.8. The dog fed the 18 per cent casein diet without pyridoxine still maintained a normal hemoglobin level, 13.0 per cent. After 100 days the normal dogs on the high and moderate protein diets had hemoglobin values close to 15 and 12 per cent respectively, but the corresponding pyridoxine-deficient groups had only 5 and 8 per cent. Thus the expected rise in hemoglobin of the normal young animals was greater on the high protein than the moderate protein diet, but the drop due to the pyridoxine deficiency was more marked in the group fed the high casein diet.

In all cases the pyridoxine-deficient dogs excreted only kynurenine and xanthurenic acid, and the normal dogs only kynurenine and kynurenic acid after ingesting the *l*(-)-tryptophane. There were no untoward reactions following the feeding of the amino acid in any of the dogs which had been on the diet only 30 days, nor in any of those fed the moderate protein diet for 65 or 100 days, except the splenectomized animals and Dog 485, which vomited a little water 8 hours after the medication. On the other hand, all the pyridoxine-deficient dogs fed the high protein diet for 100 days vomited at intervals and appeared weak and disoriented after the amino acid was administered. The severity of these reactions appeared to vary directly with the progress of the deficiency.

*Effect of Splenectomy*—Three dogs, two fed the moderate protein diet, one normal and one deficient, and a third fed the high protein pyridoxine-deficient diet, were splenectomized 26 days before the tryptophane was fed, and 66 or 74 days after they were placed on the diets.<sup>4</sup>

<sup>4</sup> The splenectomies were performed by Lillian S. Bentley.

TABLE II

*Response of Young Dogs on Normal and Pyridoxine-Deficient Diets Containing 18 or 45.8 Per Cent Protein to Administration of 1.5 Gm. of l(-)-Tryptophane*

Dog	Diet	Period on diet	Hemo-globin	Hrs. after tryptophane feeding	Urinary end-products			Comment
					Xanthur- enic acid	Kynure- nic acid	Kynur- enic acid	
		days	gm. per 100 gm blood					
490 ♂	High protein, normal	30	13.9	2-5	-	+	+++	No symptoms
				7	-	+	-	
480 ♀	High protein, pyridoxine-deficient	30	10.8	7-18	++++	+	-	" "
				19	-	-	-	
470 ♂	" "	30	10.7	7-18	++++	+	-	" "
				19	-	-	-	
486 ♂	Moderate protein, pyridoxine-deficient	30	13.0	14	++	+	-	" "
497 ♀	High protein, normal	65	17.1	2	-	+	+	" "
				3	Trace	+	++++	
				6-9	-	+	++	
				11	-	+	-	
493 ♂	High protein, pyridoxine-deficient	65	11.0	5-7	++	+	-	Vomited 4 hrs. after tryptophane
				8-11	+	+	-	
				13	-	-	-	
467 ♂	High protein, nicotinic acid-deficient; no source of unknowns	100	15.1	4-7	-	+	++	No symptoms
				9	-	-	+	
				12	-	-	-	
477 ♀	Moderate protein, pyridoxine-deficient	100	6.9	6	+	+	-	" "
				11-24	+++	+	-	
				28	-	-	-	
485 ♀	" "	100	10.7	9-13	++	+	-	Vomited 8 hrs. after tryptophane
				24	+	-	-	
				28	-	-	-	
476 ♀	High protein, pyridoxine-deficient	100	3.5	11	++++	+	-	Vomited 9 and 13 hrs. after tryptophane
				24	++++	+	-	
				28	Trace	-	-	

The hemoglobin level in all three cases fell rapidly following the operation. However, the excretion of the metabolic products of tryptophane followed

TABLE II—*Concluded*

Dog	Diet	Period on diet	Hemoglobin	Hrs. after tryptophane feeding	Urinary end-products			Comment
					Xanthuronic acid	Kynurenine	Kynuronic acid	
		days	gm per 100 gm. blood					
470 ♂	High protein, pyridoxine-deficient	100	6.6	3	++	+	—	Vomited during night after tryptophane
				8-28	++++	+	—	
				31-63	++	+	—	
				85	+	—	—	
469 ♂	Moderate protein, normal	100	11.9	4-9	—	—	+++	No symptoms
				10	—	—	—	
478 ♀	" "	100	12.2	4-6	—	+	++	Spleneetomized 26 days before tryptophane test; vomited 2 hrs. after tryptophane
				8-12	—	+	++++	
				14	—	—	—	
492 ♂	Moderate protein, pyridoxine-deficient	100	6.9	5	++	+	—	Spleneetomized 26 days earlier, vomited 1 hr. after tryptophane
				12	+	+	—	
				15-23	++	+	—	
				24	—	—	—	
489 ♂	High protein, pyridoxine-deficient	92	4.8	6	+	+	—	Spleneetomized 26 days earlier, vomited 1 and 11 hrs. after tryptophane
				7-13	+++	+	—	
				15	+	+	—	
				23	—	—	—	

the same pattern as in the other earlier experiments. Apparently the spleen has no function in regard to the disturbance of the mechanism of tryptophane utilization.

*Use of Nicotinic Acid-Deficient Diets*—Two dogs, No. 467 (Table II) and No. 441 (Table I), were maintained on the 45.8 per cent casein diet with pyridoxine and the other vitamins except nicotinic acid. They were also deprived of the fullers' earth-treated rice bran extract so that they had no source of biotin or any unknown vitamins, unless the corn-starch of the basal diet may be considered such a source. Nevertheless, these dogs grew normally and manifested few symptoms of deficiency. Their hemoglobin levels were similar to those of the control dogs and they reacted in the same way as the latter to the administration of the tryptophane. Without unknowns or nicotinic acid, dogs fed this high protein purified diet apparently withstood the deficiency for long periods. This is somewhat

like the observation of Handler and Featherston (11), who were not able in all cases to obtain typical blacktongue on purified diets containing 19 per cent casein. Schaefer, McKibbin, and Elvehjem (12) reported success in producing the nicotinic acid deficiency with purified diets, but these authors also used the low protein diet and a relatively low allowance of pantothenic acid, 0.5 mg. per kilo per day. With high protein diet, substantially free of nicotinic acid and of unknowns, but with 3 mg. of pantothenic acid per kilo per day, these dogs maintained for many months nearly normal hemoglobin levels, and exhibited no buccal erosions and no abnormality in utilization of tryptophane.

*Feeding of Xanthurenate*—In their 6th month on the diets, the pyridoxine-deficient dog, Dog 443, and the control, Dog 451, were given 250 mg. of iron xanthurenate, and 2 days later 150 mg. of pure xanthurenic acid. On both occasions the dogs excreted the xanthurenate unchanged.<sup>5</sup> No kynurenic acid was excreted in either case. In somewhat similar experiments with rats by Reid, Lepkovsky, Bonner, and Tatum (13), xanthurenic acid fed to normal animals appeared to be destroyed, since it was not excreted as such nor did kynurenic acid appear. The pyridoxine-deficient rats excreted the xanthurenic acid unchanged. There is, thus, an apparent species difference between the rat and the dog in the ability of the normal animal to destroy xanthurenic acid.

The failure of Musajo (2) to detect xanthurenic acid in the urine of dogs when they were fed a high protein diet, although he found this end-product in the urine of rats and rabbits which were similarly fed, may be due either to the instability of the product in dog urine or to his use of partially pyridoxine-deficient diets for the rats and rabbits, but not for the dogs. However, in accord with Musajo's observation, neither normal nor severely pyridoxine-deficient dogs fed the high protein diet in this study were found spontaneously to excrete xanthurenic acid. This is in contrast with the experience of Cartwright, Wintrobe, Jones, Lauritsen, and Humphreys (6) with swine. They observed kynurenic as well as xanthurenic acid in the urine of pyridoxine-deficient animals and very little kynurenic acid in that of normal animals. Moreover, the xanthurenic acid excretion occurred when a 26 per cent casein diet was fed without the tryptophane supplement.

The decisive effect of pyridoxine in determining whether kynurenic acid or xanthurenic acid shall be excreted after tryptophane feeding indicates that it plays some rôle in the reaction leading to the formation of the quinoline derivative, the presence of pyridoxine preventing the addition of the OH group at the 8 position. The significance of this is at present not

<sup>5</sup> The iron xanthurenate was prepared from the urine of pyridoxine-deficient rats as described by Reid, Lepkovsky, Bonner, and Tatum (13).

apparent. The appearance of kynurenine in the urines of both normal and deficient dogs indicates the correctness of Musajo's theory that this substance is the common intermediary between tryptophane and both kynurenic and xanthurenic acids.

The adverse effect of tryptophane feeding upon pyridoxine-deficient dogs would seem to indicate a direct relationship of pyridoxine deficiency to tryptophane metabolism, in that in such deficient dogs the administration of tryptophane seemed to precipitate a crisis. Albanese, Holt, Kajdi, and Frankston (14) reported a reduction in both plasma protein and hemoglobin in rats on tryptophane-deficient diets. Possibly the anemia of these pyridoxine-deficient dogs is related to this partial failure of tryptophane utilization.

#### SUMMARY

Young dogs, fed purified diets containing 45.8 or 18 per cent casein and ample supplements of all vitamins except pyridoxine, excreted kynurenine and xanthurenic acid but no kynurenic acid after ingestion of single doses of 2 gm. of *dl*-tryptophane or 1.5 gm. of *l*(-)-tryptophane. Similarly treated animals, given pyridoxine, excreted kynurenine and kynurenic acid but no xanthurenic acid, except traces after administration of the *dl*-tryptophane. None of these products appeared in the urines unless the tryptophane supplement was given.

The dogs excreted xanthurenic acid following the administration of tryptophane after only 30 days on the pyridoxine-deficient régime, and this abnormality continued throughout prolonged periods up to 407 days.

The more severely deficient dogs exhibited nausea, anorexia, and sometimes collapse after ingesting the tryptophane, but the normal animals and those in the earlier stages of the pyridoxine deficiency were unaffected.

The moderate protein pyridoxine-deficient diet produced the same symptoms as did the corresponding high protein diet, but somewhat less rapidly.

The high protein diet containing pyridoxine, a generous amount of pantothenic acid, and all other vitamins except nicotinic acid, and any unknowns except those present in the corn-starch used, allowed young dogs to develop normally, to maintain normal hemoglobin levels, and to metabolize tryptophane normally.

Removal of the spleen did not affect the course of tryptophane metabolism in the pyridoxine-deficient animals.

Xanthurenic acid fed to normal and deficient dogs was excreted unchanged in both cases.



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# THE ANTIRIBOFLAVIN EFFECT OF GALACTOFLAVIN

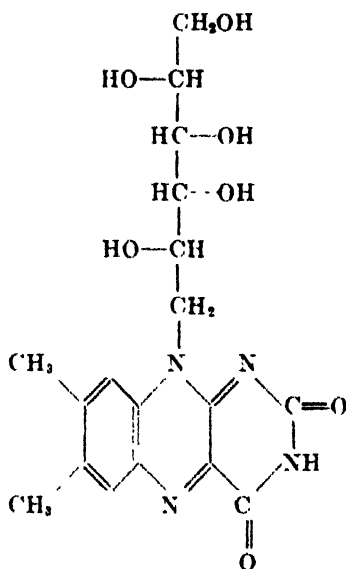
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A number of riboflavin derivatives have been shown to inhibit the utilization of this vitamin (1-3). Two of these compounds, isoriboflavin (5,6-dimethyl-9-(*d*-1'-ribityl)-isoalloxazine, 6,7-dimethyl-10-(*d*-1'-ribityl)-isoalloxazine) and the phenazine analogue of riboflavin, depressed the growth of animals receiving suboptimal levels of riboflavin, an effect that was counteracted by the feeding of an excess of the vitamin.

This communication reports the antivitamin activity of another analogue of riboflavin; namely, galactoflavin (6,7-dimethyl-9-(*d*-1'-dulcetyl)-isoalloxazine, 7,8-dimethyl-10-(*d*-1'-dulcetyl)-isoalloxazine). This compound



differs from riboflavin only in that the dulcetyl group has replaced the ribityl group as the sugar moiety of the molecule.

## EXPERIMENTAL

Weanling male rats averaging 42 gm. in weight were placed on a riboflavin-free diet, the composition of which is as follows: vitamin-free casein 18 per cent, dextrose 68 per cent, U. S. P. Salt Mixture 1, 4 per cent,

Crisco 8 per cent, cod liver oil 2 per cent; supplemented with 0.8 mg. each of thiamine and pyridoxine, 5 mg. of Ca pantothenate, 10 mg. of nicotinamide, and 100 mg. of choline chloride per 100 gm. of diet. The twelve groups employed are indicated in Table I. The galactoflavin was fed daily at a level of 1.08 or 2.16 mg. and with varying amounts of riboflavin; namely, 10, 40, 120, and 200  $\gamma$ . The 10  $\gamma$  level does not supply the rat's need for this vitamin, the young rat's requirement being about 40  $\gamma$  daily. The high levels may therefore be considered to exceed the rat's need for riboflavin. The vitamin and the inhibitor were administered by stomach tube as a suspension in gum acacia.

TABLE I  
*Effect of Galactoflavin upon Growth of Rats on Varying Intakes of Riboflavin*

Group No.		Period I		Period II*	
		No. of rats	Average gain in weight, 28 days	No. of survivors	Average gain in weight, succeeding 21 days
			gm		gm
1	Riboflavin-free	10	15	10	
2	1.08† mg. galactoflavin	10	2	9	
3	2.16 " "	9	-1	3	
4	10 $\gamma$ riboflavin	9	75	9	
5	10 " " + 2.16 mg. galactoflavin	9	11	7	68
6	40 " "	9	102	9	
7	40 " " + 2.16 mg. galactoflavin	9	26	9	75
8	120 " "	10	106	10	
9	120 " " + 1.08 mg. galactoflavin	10	102	10	
10	120 " " + 2.16 " "	10	75	10	62
11	200 " "	10	118	10	
12	200 " " + 2.16 mg. galactoflavin	10	93	10	

\* Riboflavin increased to 200  $\gamma$  daily between Periods I and II.

† Equimolar equivalent of 1 mg. of riboflavin.

Galactoflavin had an inhibiting effect upon the growth of rats maintained on the riboflavin-free diet (Groups 2 and 3 as compared with Group 1); furthermore, the feeding of the higher level of the drug had a marked influence on survival; six of the original nine rats had succumbed by the 26th day of the test. The depressing effect upon growth was also observed with the rats receiving the lower levels of riboflavin (10  $\gamma$  and 40  $\gamma$ ) (Groups 5 and 7, Period I, as compared with Groups 4 and 6). The inhibitor was fed only at the higher level. The riboflavin was increased from 10 and 40  $\gamma$  to 200  $\gamma$  between Periods I and II, with a resulting stimulus in the growth response. When the inhibitor was fed at a level of 1.08 mg.

daily in conjunction with 120  $\gamma$  of riboflavin, the increment in weight was approximately the same as for the controls (Group 9 as compared with Group 8); however, the feeding of the higher level of the drug (2.16 mg.) depressed growth (Group 10 as compared with Group 8, Period I). This effect was not as marked as that observed with the same quantity of galactoflavin and the lower level of riboflavin. Increasing the riboflavin intake from 120 to 200  $\gamma$  between Periods I and II resulted in only a slight augmentation in the rate of growth.

The growth-depressing effect incurred by the feeding of 2.16 mg. of galactoflavin was not completely prevented by the daily administration of 200  $\gamma$  of riboflavin (Group 12 as compared with Group 11). This lack of complete counteraction may perhaps be explained on the basis of solubility. It is possible that the riboflavin might have been better utilized had it been administered in the diet rather than in a single dose.

#### SUMMARY

Galactoflavin, when fed to rats on a riboflavin-free diet, suppressed growth beyond that attributable to the deficiency of riboflavin alone; furthermore, the mortality rate was increased. The compound likewise inhibited the growth of rats receiving low levels of riboflavin, an effect that was almost completely counteracted by the feeding of excess riboflavin.

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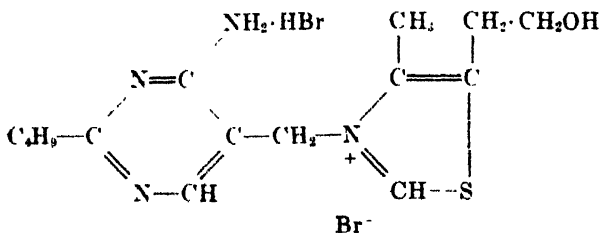
## THE ANTITHIAMINE EFFECT OF THE 2-*n*-BUTYLPYRIMIDINE HOMOLOGUE OF THIAMINE

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The antivitamin activity that may result from the feeding of structurally altered vitamins has been discussed by Woolley (1). Pyriethiamine, the pyridine analogue of thiamine, was found by Robbins (2) to inhibit the growth of certain fungi. The antithiamine effect of pyriethiamine was demonstrated in mice by Woolley and White (3), who were able to produce the characteristic signs of thiamine deficiency by the feeding of this compound. Furthermore, the deficiency state so induced could be prevented or cured by sufficient amounts of thiamine. The antithiamine activity of the 2-*n*-butylpyrimidine homologue of thiamine (2-*n*-butyl-5-(4-methyl-5-*p*-hydroxyethyl-thiazolium bromide)-methyl-6-aminopyrimidine hydrobromide) is herein reported. The structure of this compound is as follows:



Preliminary tests demonstrated that this compound was devoid of thiamine activity and apparently functioned as a thiamine inhibitor. Accordingly an experiment was designed to establish this effect.

Forty-eight weanling rats divided into six groups of like average weight were placed on a thiamine-deficient diet consisting of technical casein 16 per cent, corn-starch 60 per cent, autoclaved yeast 9 per cent, salt mixture (U. S. P. No. 1) 4 per cent, hydrogenated vegetable oil 9 per cent, cod liver oil 2 per cent, and non-autoclaved brewers' yeast 0.2 per cent.

The animals received supplements daily by stomach tube as follows: Group 1, none; Group 2, 2.8 mg.<sup>1</sup> of the 2-*n*-butylpyrimidine homologue of thiamine (bromide hydrobromide); Group 3, 5  $\gamma$  of thiamine; Group 4,

<sup>1</sup> Equimolar equivalent of 2 mg. of thiamine (chloride hydrochloride).

5  $\gamma$  of thiamine and 2.8 mg. of the homologue; Group 5, 50  $\gamma$  of thiamine; Group 6, 50  $\gamma$  of thiamine and 2.8 mg. of the homologue.

The feeding of the thiamine homologue decreased the survival period of rats maintained on a thiamine-low diet (Table I, Groups 1 and 2). The most striking effect, however, was noted when the compound was fed in conjunction with a suboptimal level of thiamine (5  $\gamma$ ). Under these conditions the preparation had a marked depressing effect upon growth. Moreover, all animals in the group developed polyneuritis, five out of eight succumbed, and the remaining three were moribund after 50 days on the test. Rats receiving the same intake of thiamine, but without the homologue, made continuous gains, were free from polyneuritis, and

TABLE I  
*Response of Rats to Thiamine and 2-n-Butylpyrimidine Homologue of Thiamine*

Group No. (Eight rats each, males)		Average gain in weight, 30 days	No. of survivors	Average gain in weight, 50 days	No. of survivors	Average length of survival	Remarks
		gm.		gm		days	
1	Control	21	8		0	38	
2	2.8 mg. 2- <i>n</i> -butylpyrimidine homologue of thiamine	23	3		0	29	
3	5 $\gamma$ thiamine	86	8	117	8		No polyneuritis All animals showed polyneuritis
4	5 " " + 2.8 mg. 2- <i>n</i> -butylpyrimidine homologue of thiamine	51	8	37	3		
5	50 $\gamma$ thiamine	130	8	193	8		
6	50 " " + 2.8 mg. 2- <i>n</i> -butylpyrimidine homologue of thiamine	114	8	166	8		

all survived the 50 day test period (Groups 3 and 4). The true antithiamine nature of the compound was demonstrated by the fact that the growth-depressing effect of the inhibitor was almost completely prevented by the administration of excess thiamine (50  $\gamma$ ) (Groups 5 and 6). These findings indicate that 1 mole of thiamine counteracts the antithiamine effect of about 40 moles of the homologue, an antithiamine activity comparable with that reported by Woolley and White (3) for pyrithiamine.

It is of interest to note that the 2-ethyl derivative of thiamine possessed full thiamine activity when tested with rats (4), and the 2-*n*-propyl analogue showed some activity in assays with pigeons (5). The 2-*n*-butyl homologue was not only inactive but acted as an antivitamin.

## SUMMARY

The administration of the 2-*n*-butylpyrimidine homologue of thiamine to rats maintained on a suboptimal intake of thiamine produced polyneuritis and subnormal growth, characteristics of thiamine deficiency. These effects were nullified by the feeding of excess thiamine.

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# ACETYLATION OF SULFANILAMIDE BY LIVER HOMOGENATES AND EXTRACTS\*

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The acetylation of sulfanilamide, discovered by Marshall *et al.* (1) and by Fuller (2), has been the subject of a large number of investigations. Since a considerable fraction of the drug may circulate and be excreted in acetylated form, the phenomenon has practical importance (Stewart, Rourke, and Allen (3)). Furthermore, the reaction has been used frequently in studies of the mechanism of acetylation (4-6), in which the easily determined sulfanilamide serves as a trap for acetyl groups.

Acetylation of sulfonamides has been observed in men and in a large number of mammals, except the dog, in birds, and also in cold blooded animals (Marshall (7)). Generally the liver was found to be the sole site of acetylation (8); in the cat, other organs do, however, seem to participate in the reaction (9). Acetylsulfanilamide, once formed, is not decomposed again in the animal body.

The studies mentioned so far were carried out with intact animals or by perfusion of isolated organs. Klein and Harris (10), however, demonstrated the applicability of an *in vitro* technique to the study of sulfanilamide acetylation. Their experiments with rabbit liver slices contributed considerably to the clarification of the mechanism of this reaction. Particularly noteworthy was the demonstration of a coupling between acetylation and respiration.

It was the purpose of the present investigation to approach more closely the study of the enzymatic mechanism of acetylation. The primary problem, therefore, was to prepare active cell-free preparations. Attempts by Klein and Harris to obtain acetylation in ground up rabbit liver had given negative results. With a more cautious procedure we obtained some acetylation in rabbit liver homogenate. Better, and sometimes even abundant, acetylation was found with preparations of pigeon liver, first with homogenates and, later, with extracts and acetone preparations. Through the use of these enzyme preparations it has been possible to advance further the analysis of the mechanism of sulfanilamide acetylation. Particularly, the coupling with energy-yielding reactions could be traced to a dependence on the supply of energy-rich phosphate bonds through adenylyl pyrophosphate.

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### Methods

*Sulfanilamide*—The acetylation of sulfanilamide was followed by the method of Bratton and Marshall (11) by which only free sulfanilamide is directly determined. As described by Bratton and Marshall, acetylated sulfanilamide may, however, be determined as the increase in free sulfanilamide after hydrolysis with 0.2 N hydrochloric acid in boiling water.

It soon was found consistently that the originally added sulfanilamide was quantitatively recovered after acid hydrolysis. This indicated that in our procedure destruction of sulfanilamide was negligible and that its disappearance could safely be attributed to conjugation (*cf.* Table II). Therefore acid hydrolysis was frequently omitted and conjugation was determined as the difference between the control and incubated sample. In the important experiments, however, checks by acid hydrolysis were made on the recoverability of disappearing sulfanilamide.

Throughout the experiments trichloroacetic acid was used for deproteinization in such amounts as to give a final concentration of 3 to 4 per cent.

*Tissue Preparation*—For the preparation of homogenates, essentially the directions of Potter and Elvehjem were followed (12). The dimensions of the homogenizer were adapted to accommodate fluid volumes up to 50 ml. A glass plunger was prepared to fit the average width of 25 × 200 mm. Pyrex test-tubes, and from a large number of such tubes a series of slightly different widths was chosen. These were graded arbitrarily from No. 1 to No. 4, No. 1 being quite tight fitting and No. 4 allowing an interspace of about 1 mm. between the wall and the plunger.

One or two pigeon livers, weighing 7 to 10 gm. each, were finely minced with stainless steel scissors. The minced tissue was suspended in 1 to 1½ times its volume of a fluid which in composition approximates the intracellular salt milieu. A formula was used similar to that introduced by Buchanan, Hastings, and Nesbitt (13) in their experiments on glycogen synthesis in liver slices. The solution contained 0.13 M potassium chloride and 0.01 M magnesium chloride, and was buffered with either phosphate, pH 7.6, or bicarbonate, in a 0.03 to 0.04 M concentration.

The relatively coarse suspension of liver particles was first homogenized in a loose fitting tube, No. 3 or No. 4. Connective tissue was removed by filtration through two layers of wide meshed gauze. The filtrate was further homogenized in a tight fitting tube. During these operations the preparation was kept cold by packing the tube in crushed ice. If not stated otherwise, the homogenates were used immediately after preparation.

These preparations contained approximately 0.4 gm. of liver per ml.

of homogenate. In spite of such a high concentration of liver pulp, the homogenate was rather fluid and could easily be pipetted when the connective tissue had been removed by filtration through gauze.

#### EXPERIMENTAL

*Acetylation in Homogenates of Liver; Outline*—The first tests were made with rabbit liver which had been used in the tissue slice experiments of Klein and Harris (10). The results were encouraging in a qualitative

TABLE I

*Respiration, Phosphate Turnover, and Sulfanilamide Acetylation of Pigeon Liver Homogenate*

Each respiration vessel contained 1 ml. of homogenate, prepared as described, plus 0.5 ml. additions, and NaOH in the center cup. The vessels were shaken with air as the gas phase in a thermostat at 37°. The experimental period is counted from immersion into the thermostat. The experiment was terminated by the addition of 3 ml. of 5 per cent trichloroacetic acid after 1½ hours incubation. The data for phosphate and sulfanilamide refer to the total incubation period.

Addition*	Oxygen consumption		P turnover		Sulfanilamide acetylation	
	Second 10 min.	Following 30 min	Experiment	Experiment minus control†	Experiment	Experiment minus control‡
	c mm.	c mm.	ml.	mg	γ	γ
0	80	241	1.31	+0.45	13	-53
Acetate, 0.025 M	99	292	1.27	+0.41	11	-55
Pyruvate, 0.025 M	86	210	1.29	+0.43	10	-56
Same + malonate, 0.02 M	105	271	1.21	+0.35	15	-51
Fumarate, 0.02 M	96	249	1.26	+0.40	18	-48
Same + fluoride, 0.02 M	89	270	0.55	-0.31	11	-55

\* The acids were added as their sodium salts.

† Control, 0.86 mg. of P before incubation

‡ Control, 66 γ; the added sulfanilamide, measured in a control without incubation.

manner; a definite although rather slight acetylation was observed with rabbit liver homogenate. For instance, when 67 γ of sulfanilamide were added to 1 ml. of homogenate and incubated aerobically at 37° for 60 minutes, 15 per cent was conjugated with added acetate and 8.5 per cent without acetate. For the contemplated study of the finer mechanism of acetylation, however, such an activity did not seem satisfactory, especially since less or no acetylation was frequently found with homogenized rabbit liver. Rat liver was tested next and, in accordance with the experience of earlier workers (10), was found still less suitable for *in vitro* experiments. Eventually we succeeded in the search for active cell-free

material by finding in homogenates of pigeon liver a vigorous acetylation of sulfonamides.

In Table I some characteristics of this preparation are surveyed. The oxygen consumption amounts to somewhat less than half the values reported by Evans (14) with pigeon liver suspension prepared with a Latapie grinder, and remained steady for at least 1 hour. A considerable breakdown of cellular phosphate compounds occurred during incubation without fluoride, while with fluoride appreciable phosphate fixation was observed. When 66  $\gamma$  of sulfanilamide were added, 80 per cent was acetylated even without the addition of a metabolic substrate. Therefore the differences in acetylation due to added acetyl donors were not well pronounced in this experiment. Notable, nevertheless, is an increase in acetylation with fluoride, which parallels the positive phosphate balance (last two lines of Table I). Such an effect of fluoride was not always pronounced. It was particularly well observed with slight acetylation (*cf.* Table VI). However, this early indication of a connection between acetylation and phosphorylation lead eventually to an isolation of the essential enzyme system, in which, independent of respiration, adenylyl pyrophosphate acting as an energy donor was found to promote the conjugation of sulfanilamide. In the following paragraphs, first the over-all process will be described as it occurs in fresh homogenate, dependent largely on respiratory energy supply. Subsequently some properties of the isolated enzyme system will be described.

*Coupling between Respiration and Acetylation*—To study the influence of various factors the experimental conditions had to be adjusted to maintain a steady saturation of the enzyme system throughout the experiment. For this purpose, it was necessary to shorten the incubation time as well as to increase the concentration of sulfanilamide. In Table II the results of an experiment are recorded in which aerobic and anaerobic acetylation were followed with the addition of various metabolites. To aerobic samples 630  $\gamma$  of sulfanilamide were added per ml. of homogenate and the incubation was continued for 15 minutes only. To follow accurately the lesser, anaerobic effect, less sulfanilamide was added and the incubation time extended to half an hour.

The data of Table II show the following characteristics. As an acetyl donor, acetate is certainly superior to all other compounds tested. This observation parallels earlier results with sliced liver tissue (10) and with intact animals (15, 16, 5). The addition of acetate increased acetylation by 88 per cent. A pronounced, though smaller, increase of 48 per cent is seen with pyruvate and of 40 per cent with acetoacetate. Slighter increases were found with acetoin and diacetyl<sup>1</sup> bearing out earlier observations with intact animals (17). The apparent ease with which ace-

<sup>1</sup> Dr. W. W. Westerfeld kindly supplied samples of these compounds.

toacetate functions as an acetyl donor is remarkable. Although so many indications point toward an equilibrium between acetate and acetoacetate (18, 19), a chemical definition of this reaction has not yet been possible.

Secondly the data evidence the ultimate dependence of abundant acetylation on continuous energy supply by the 5-fold aerobic increase in acetylation over and above the anaerobic level, even when the greater time of anaerobic incubation is not taken into account. Nevertheless, a consistent residual anaerobic acetylation remained which may be influenced by the addition of the same acetyl donors as those which activate

TABLE II

*Aerobic and Anaerobic Sulfanilamide Conjugation: Effect of Metabolites*

Samples of 1 ml. of homogenate (0.4 gm. of liver tissue + 0.6 ml. of a solution containing 0.7 per cent KCl, 0.01 M  $\text{MgSO}_4$ , and 0.03 M  $\text{K}_2\text{HPO}_4$ ) in a total volume of 1.4 ml. were shaken at 37° in Warburg vessels in an atmosphere of either air or nitrogen.

The respiratory activity of this preparation was comparable to that used in the experiments of Table I.

Substrate added, final concentration	Aerobic				Anaerobic			
	Incubation time	Sulfanilamide			Incubation time	Sulfanilamide		
		Direct	After hydrolysis	Acetylated		Direct	After hydrolysis	Acetylated
		(1)	(2)	(2) - (1)		(1)	(2)	(2) - (1)
	min.	$\gamma$	$\gamma$	$\gamma$	min.	$\gamma$	$\gamma$	$\gamma$
	0	630	630	0	0	442	440	0
0	15	495	640	145	30	420	452	32
0.025 M acetate		370	642	272		379	455	76
0.04 " acetyl phosphate						400	446	46
0.018 " acetoacetate		412	615	203				
0.025 " pyruvate		436	650	214		387	443	56
0.013 " diacetyl		478	625	147		393	437	44
0.013 " acetoin		474	650	176				

aerobically. In the anaerobic experiment, moreover, acetyl phosphate was tried as an acetyl donor. Its relatively slight effect will be discussed in a later paragraph in connection with experiments on the utilization of phosphate bond energy in the process of acetylation.

*Acetate Effect*—Further details on the rate of acetylation and on its activation through acetate are given in Table III. An experiment with and without acetate on a larger scale is represented in Fig. 1. Quite generally an addition of acetate increased the rate of acetylation by 50 to 100 per cent; occasionally, however, little or no effect was observed, for instance in the first experiment of Table IV.

In order to account in terms of acetate for such acetylation as occurs

without an added substrate, the steam-volatile acid was determined in several samples of homogenate. Per ml. of homogenate levels of 1.5 to 2 micromoles were found, increasing little during incubation. Such an amount of acetate theoretically may account for an acetylation of 260 to 340  $\gamma$  of sulfanilamide. Actually the maximal acetylation without a substrate amounted to only 172  $\gamma$ . The acetate balance becomes even more favorable if it is considered that acetate presumably is oxidized as well as produced during the experiment, since its addition increases oxygen consumption (Table I).

TABLE III

*Effect of Acetate on Aerobic Sulfanilamide Conjugation*

The experimental procedure was the same as in the aerobic experiment of Table II.

Experiment No.	Incubation time	Sulfanilamide conjugated per ml. homogenate		Remarks
		No. addition	50 micromoles acetate added	
	<i>min</i>	$\gamma$	$\gamma$	
1	10	58	108	501 $\gamma$ sulfanilamide were added at start of experiment
	20	120	230*	
	30	164	316	
	50	172	346	
2	30	43	69	420 $\gamma$ sulfanilamide added at start
	60	47	112	
	120	51	127†	

\* In a parallel anaerobic experiment 76  $\gamma$  of sulfanilamide were conjugated with either acetate or acetyl phosphate added.

† Steam-volatile acid was determined, directly after the acetate addition, as 52 micromoles and at the end of the experiment as 58 micromoles per ml. of homogenate.

*Relative Rates of Acetylation with Various Sulfonamides*—In Table IV the relative extent of acetylation *in vitro* was determined with some of the commonly used sulfonamides. The highest yield of acetyl compound was given by sulfanilamide, the lowest by sulfadiazine. The same sequence appears in data for acetylation in the human body compiled by Janeway (20).

*Activation of Acetylation by Cocarboxylase in Thiamine-Deficient Liver Preparations*—In the experiments of Table V the livers of pigeons were used which had been kept on polished rice until deficiency symptoms appeared. Here, the addition of cocarboxylase to the homogenate increased acetylation, in particular with added acetate. The effect was well pronounced only in Experiment 1. Deficiency experiments were complicated by a frequent disappearance of the ability to acetylate sulfonamides in livers of strongly deficient birds. The effect of thiamine on

acetylation in the intact animal had previously been observed by Martin *et al.* (21) with deficient rats.

In the deficient system cocarboxylase causes the greatest increase of acetylation with excess of acetate. Therefore it seems that in the thiamine-

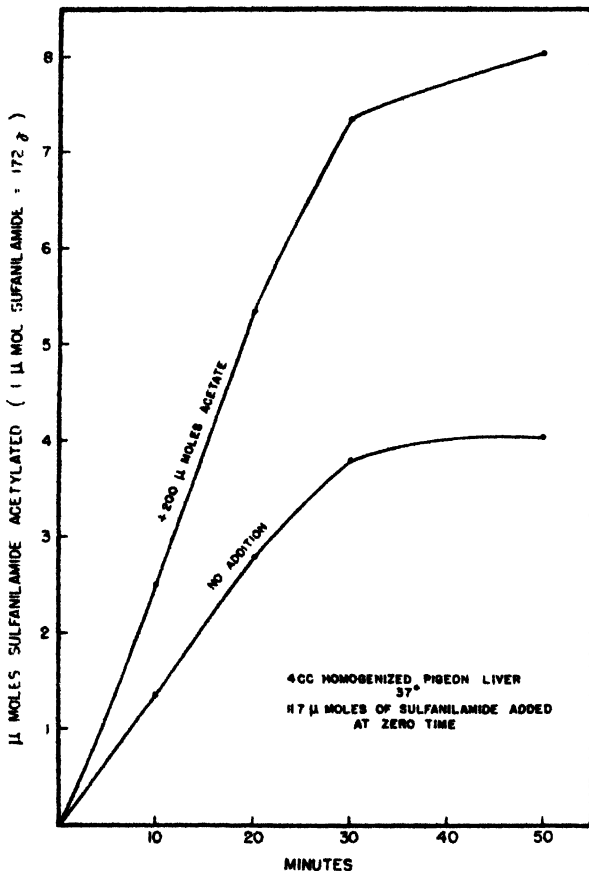


FIG. 1. Time curve of acetylation and the effect of acetate. Experimental conditions were similar to those described in Table II, except that Erlenmeyer flasks were used instead of Warburg vessels. The flasks were shaken in the thermostat with air as the gas phase, and at the indicated times samples were taken for analysis.

deficient system the process of conjugation rather than the availability of the acetyl group is inhibited. When this finding is related to the dependence of acetylation on a supply of energy-rich phosphate bonds, subsequently described, the observed activation of acetylation by cocarboxylase may be attributed to an increased supply of phosphate bonds, thus indirectly implying an inefficient coupling between oxidation and phos-



phorylation in the thiamine-deficient system (22). Likewise, insulin had been found to increase acetylation considerably (23), while recent experiments with radioactive phosphate show that insulin intensifies the turnover of adenylyl pyrophosphate in the liver of the rat (24).

TABLE IV

*Acetylation of Various Sulfonamides and of p-Aminobenzoic Acid by Liver Homogenate*

Samples of 1 ml. of homogenate, in a total volume of 1.3 ml., were shaken in Warburg vessels with air at 37°. The incubation time was in all cases 20 minutes. To facilitate a comparison of the rate of conjugation, the data are given in micromoles per ml. of homogenate. 2.5 micromoles of the compound in each case were added at the start.

Compound	Acetate added	Sulfonamide conjugated
	micromoles*	micromoles*
Sulfanilamide	0	1.00
	50	1.09
Sulfathiazole	0	0.30
	50	0.40
Sulfadiazine	0	0.20
	50	0.30
p-Aminobenzoic acid	0	0.64
	50	0.62

\* 1 micromole of sulfanilamide = 172  $\gamma$ .

TABLE V

*Effect of Cocarboxylase on Acetylation in Homogenates of Thiamine-Deficient Pigeon Liver*

Samples of 1 ml. of homogenate (0.4 gm. of fresh tissue + 0.6 ml. of 0.13 M KCl, 0.02 M  $MgCl_2$ , and 0.04 M  $NaHCO_3$ ) in 1.35 ml. of total volume were shaken in Warburg vessels at 37° in an atmosphere of oxygen-5 per cent  $CO_2$  for 45 minutes. 345  $\gamma$  of sulfanilamide were added at the start of the experimental period.

Experiment No.	Acetate	Sulfanilamide conjugated	
		No addition	16 $\gamma$ cocarboxylase
1	micromoles	$\gamma$	$\gamma$
	None	40	55
	50	83	124
2	None	50	52
	50	50	58

*Coupling between Phosphorylation and Acetylation*—In the experiments of Table I some increase of acetylation with fluoride was noted. The data of Klein and Harris (10) suggested a positive effect of fluoride. Although they stated merely that fluoride does not inhibit acetylation, their data indicated a slight increase with fluoride. In the experiment of Table VI

TABLE VI  
*Effect of Fluoride and Adenyl Pyrophosphate*

Each vessel contained 1 ml. of fresh homogenate with 0.03 M sodium bicarbonate in a total volume of 1.25 ml. 60 minutes incubation at 36° after dipping in 57 γ of sulfanilamide. The gas space of the Warburg vessels was filled with O<sub>2</sub>-5 per cent CO<sub>2</sub> or N<sub>2</sub>-5 per cent CO<sub>2</sub> respectively.

Addition	Conjugated sulfanilamide	
	Aerobic	Anaerobic
	γ	γ
	6	
0.025 M fluoride	11	4
Same + 0.32 mg adenyl pyrophosphate-P <sub>7</sub> *		17

\* P<sub>7</sub> stands for phosphorus, hydrolyzable by 7 minutes boiling with N hydrochloric acid. All adenyl pyrophosphate was decomposed at the end of the experiment.

TABLE VII  
*Anaerobic Conjugation through Adenyl Pyrophosphate*

Samples of 1 ml. of homogenate, with 0.03 M sodium bicarbonate, were incubated at 37° in Warburg vessels filled with N<sub>2</sub>-5 per cent CO<sub>2</sub>, which had been passed over heated copper oxide. All vessels contained acetate in 0.02 M final concentration and 90 γ of sulfanilamide were added at the start with the sodium fluoride. The adenyl pyrophosphate was added from two separate annexes in equal portions, the first at the start and the second after 15 minutes incubation. The total incubation time was 30 minutes.

Experiment No.	NaF <sup>†</sup> <i>mole per l.</i>	Adenyl pyrophosphate		Sulfanilamide conjugated γ	Remarks
		Added mg. P <sub>7</sub>	Remaining mg. P <sub>7</sub>		
1	0.08	0.32	0.16	49	Fresh homogenate
	0.08	0	0	9	
	0.02	0.32	0.09	37	
	0.02	0	0	5	
2	0.02	0.32	0.08	57	Same, after freezing overnight
3	0.02	0	0	15	Different homogenate
	0.02	0.32	0	28	
	0.02	0.32	0	19*	

\* No acetate was added to this sample.

the activation by fluoride appears more distinctly, presumably because of a lower activity of this preparation.<sup>2</sup>

<sup>2</sup> A considerable decrease in the activity of liver preparations was usually observed with prolonged cage life of the animals. The highest activities were obtained with livers of pigeons which had been freshly delivered from an out of town dealer.

The assumption that the activation by fluoride may be due to a protection of energy-rich phosphate bonds is borne out by the even greater effect of adenylyl pyrophosphate, although it was assayed in the absence of oxygen. More data on acetylation through adenylyl pyrophosphate are given in Table VII. Of special interest is the effect of freezing which generally decreases respiration of liver homogenates considerably. The acetylation with adenylyl pyrophosphate, however, was slightly enhanced after freezing. The increase found with the addition of acetate (Experiment

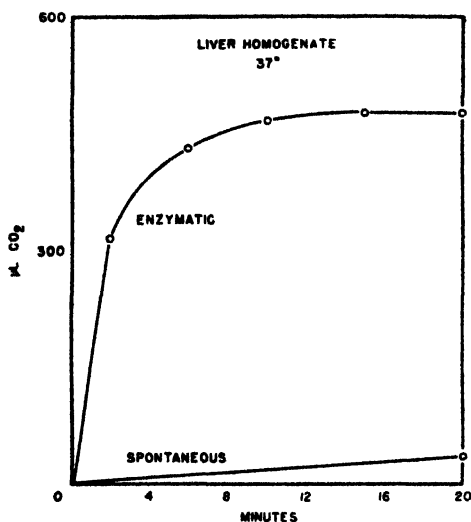


FIG. 2. Manometric measurement of enzymatic decomposition of acetyl phosphate. Samples of 1 ml. of homogenate, in a final volume of 1.65 ml., containing 0.03 M sodium bicarbonate, were shaken in Warburg vessels with N<sub>2</sub>-5 per cent CO<sub>2</sub> as the gas phase. The control contained the same amount of plain bicarbonate. In both vessels 0.3 ml. of a 0.2 M solution of sodium acetyl phosphate was dipped from an annex at zero time. The readings were not corrected for retention and the measured gas evolution, due to the acid formation with acetyl phosphate splitting, is only proportional but not equivalent to the decomposed amounts.

3 of Table VII) furnishes evidence that the conjugation brought about by adenylyl pyrophosphate is in fact acetylation.

In contrast to the strong anaerobic acetylation with adenylyl pyrophosphate, the addition of acetyl phosphate, as mentioned already, had little effect, not exceeding that of free acetate (Tables II and III). It remains to be considered that acetyl phosphate is very quickly destroyed by liver homogenate, much faster than is adenylyl pyrophosphate. Data on the enzymatic hydrolysis of acetyl phosphate in liver homogenate are reproduced in Figs. 2 and 3. In the experiment of Fig. 2, acid formation was

measured manometrically in a bicarbonate medium. In an analogous experiment the liberation of inorganic phosphate was determined by the method of Lipmann and Tuttle (25). Parallelism between acid formation and phosphate liberation shows the reaction to be a hydrolytic split of acetyl phosphate. The enzyme responsible for this reaction apparently is ubiquitous in animal tissues. It has been observed so far in extracts of kidney, brain, liver, and muscle; it is particularly abundant in muscle, and the muscle enzyme has been studied in some detail. It was found to

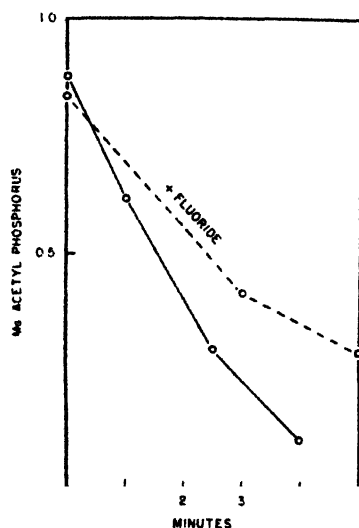


FIG. 3. Disappearance of acetyl phosphate in liver homogenate due to enzymatic decomposition. Samples of 1 ml. of homogenate containing 0.05 M sodium bicarbonate were brought into a series of test-tubes and equilibrated in a water bath of 37°. At zero time 0.85 mg. of acetyl phosphorus was added as the sodium salt. At the indicated times 5 ml. of 3 per cent trichloroacetic acid were added to one of the samples and acetyl phosphate was determined immediately. The fluoride concentration in the experiment with fluoride was 0.05 M.

be a heat-resistant, very soluble protein, probably of basic nature, the properties of which will be described in a subsequent publication.

The presence of this enzyme, although by itself of metabolic interest, tends to interfere with attempts to evaluate results obtained with acetyl phosphate in animal tissues. With a concentrated homogenate, as is needed for acetylation, acetyl phosphate has a half lifetime of a little less than 2 minutes and none is left after a 10 minute period. Fluoride increased the half lifetime by about 50 per cent, but even then very little acetyl phosphate remains after 10 minutes incubation (Fig. 3). Acetyl phosphate, however, remained a poor acetyl donor, even when during the first 15 min-

utes aerobically an abundant acetylation was observed (Table II). Such a strongly active enzyme preparation should have shown an effect in spite of rapid decomposition.

*Non-Enzymatic Acetylation of Aniline with Acetyl Phosphate*—In the enzymatic experiments with acetyl phosphate the possibility had to be considered that the acid anhydride, acetyl phosphate, might react non-enzymatically with aromatic amines. Tests were made under conditions analogous to those in the enzyme experiments, but did not show measurable non-enzymatic acetylation of sulfanilamide. An appreciable reaction was found, however, with aniline, which could be used in higher concentrations. This observation is considered to have some bearing on the problem of enzymatic acetylation, and data on the non-enzymatic acetylation of aniline with acetyl phosphate are included here.

Aniline was determined by the diazotization method used for sulfanilamide determination. When in analogy with enzyme experiments, low concentrations, for instance 1 micromole of sulfanilamide or aniline per ml., were treated with 100 micromoles of acetyl phosphate, no decrease of the free amine was observed. Positive results were obtained, however, by reversing the concentrations of the reactants and determining acetyl phosphate (25) rather than aniline. 2.5 micromoles of acetyl phosphate were incubated at 37° with and without aniline. With 0.8 per cent aniline, after 1 hour, 65 per cent of the acetyl phosphate was found decomposed against a spontaneous decomposition of only 15 per cent in the same period. This excess decomposition was attributed to the reaction



To prove this reaction the experiment was repeated on a somewhat larger scale in order to isolate acetanilide. Two samples of 2.5 per cent aniline in 10 ml. of 0.03 M propionate buffer of pH 5.8, one containing 56 micromoles of acetyl phosphate, were incubated for 1 hour at 37°. Due to the action of the aniline, 58 per cent, or 28 micromoles, of acetyl phosphate was decomposed. A separation of acetanilide was now carried out in the following manner. Both samples were extracted three times with ether in a separatory funnel. The ether extract was shaken repeatedly with 1 per cent sulfuric acid to remove the excess aniline and washed with water. Both ether extracts were now evaporated to dryness. The extract from the sample containing acetyl phosphate left on evaporation a layer of white crystals, while the control evaporated without visible residue. The residues were dissolved in 1 ml. of ethanol and made up to volume with water. Aniline was determined colorimetrically before and after 60 minutes hydrolysis at 100° with 0.2 N hydrochloric acid, by which

procedure acetanilide is hydrolyzed. Before hydrolysis, both the experimental sample and the control gave a slight color corresponding to 2 micromoles of free aniline. In the sample containing acetyl phosphate the hydrolysis liberated 22 micromoles of aniline; while in the control no increase was observed. Another aliquot was used for steam distillation. After gently refluxing in strong sulfuric acid for 50 minutes to decompose the acetanilide, 18.4 micromoles of steam-volatile acid were obtained from the solution of the crystalline residue. Considering the fact that not too great care had been taken to conduct the extractions in a rigidly quantitative manner, the approximate equivalence between decomposed acetyl phosphate, conjugated aniline, and steam-volatile acid agrees with a stoichiometric reaction between acetyl phosphate and aniline according to Equation 1.

TABLE VIII

*Preliminary Fractionation of Homogenate*

The experimental conditions were analogous to those described in Table V, except that the incubation time was 36 minutes. The homogenate was prepared the day before and was kept frozen overnight. Further details of preparation are described in the text.

Fraction	Added adenylyl pyrophosphate	Conjugated sulfanilamide
	mg $P_i$	$\gamma$
Whole homogenate	0.32	65
	0	5
Supernatant fluid	0.32	69
Particulate matter	0.32	39

*Preparation and Characteristics of Homogeneous Enzyme Solutions*—The enzymatic principle which brings about the acetylation of sulfanilamide with adenylyl pyrophosphate as a condensing agent is not bound to cell structures. From the data of Table VIII it appears that on centrifugation the activity remains in the supernatant solution. In the experiment described the frozen homogenate was thawed and centrifuged in an angle centrifuge at 4000 to 5000 R.P.M. in a cold room at 7° outside temperature. After about 20 minutes centrifugation a separation had occurred into a reddish brown, somewhat cloudy supernatant, a thick semifluid interphase, and a sediment. The supernatant, amounting to a little less than two-thirds of the original volume, was sucked off with a pipette. The residue, including interphase, was resuspended in saline, and brought back to the original volume. The particles were not washed and thus retained a little more than one-third of the fluid extract. The data show that no or little activity is bound to the sediment.

The subsequent experiments were done with particle-free solutions. As shown in Table IX, these solutions do not respond any more to oxygenation. The effect of adenylyl pyrophosphate is roughly proportional to the initial concentration, although little increase of acetylation occurred after 20 minutes incubation, presumably because of the rapid enzymatic hydrolysis of adenylyl pyrophosphate.

Preliminary observations indicate a heat-resistant component to be a part of the enzyme system. The heat-stable principle is present in larger amounts in liver; kidney and muscle contained less, and results with yeast have been negative so far. In the experiments described in Table X a reversible inactivation was brought about by keeping extracts overnight at 7°, with or without dialysis. Likewise, partial inactivation occurred on 2 hours incubation at 37°. Frequently the reactivation by

TABLE IX  
*Variation of Adenylyl Pyrophosphate Concentration and of Length of Incubation Time*

The experimental conditions were analogous to those described in Table V. At the start of the experiment 129  $\gamma$  of sulfanilamide, adenylyl pyrophosphate, and sodium fluoride were added to 0.07 M final concentration.

Incubation time	Gas phase	Adenylyl pyrophosphate	Conjugated sulfanilamide
<i>min.</i>		<i>mg. P<sub>i</sub></i>	$\gamma$
40	O <sub>2</sub> -5% CO <sub>2</sub>	0	0
20	N <sub>2</sub> -5% "	0.32	46
40	N <sub>2</sub> -5% "	0.32	55
40	N <sub>2</sub> -5% "	0.16	31

boiled liver preparation was incomplete, indicating an additional inactivation of the residual component. Rather concentrated preparations of the boiled organs had to be used for reactivation. The best results were obtained with undiluted boiled juices, prepared by heating in boiling water the mashed tissue without added fluid. No known substance has been found so far to activate the enzyme. Glutathione, up to 0.04 M, glutamate, and citrate were tried unsuccessfully. These compounds had been found by Nachmansohn *et al.* (26) to activate the enzymatic acetylation of choline. In other respects the enzymatic mechanism of sulfanilamide acetylation appears strikingly similar to choline acetylation in which Nachmansohn and Machado (27) likewise observed a linking between phosphorylation and acetylation.

Experiments in progress have shown that the enzyme remains active on treatment of extracts and of liver pulp with acetone. Extracts of acetone preparations are of equal activity or even superior to the ex-

tracts of frozen liver described above. Such preparations will prove most suitable for a further clarification of the enzymatic mechanism of sulfanilamide acetylation now in progress.

TABLE X

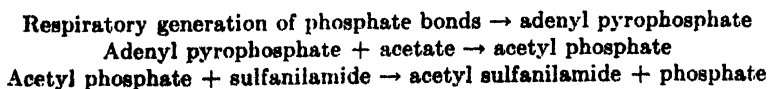
*Reversible Inactivation through Dialysis or Autolysis*

The extract, treated or untreated, was incubated in open narrow test-tubes in a water bath of 37°. The tubes contained 1 ml. of extract in a total volume of 2 ml.; magnesium chloride and sodium acetate were present in 0.02 M concentration. The extract was combined with the activator, and, after adaptation to the bath temperature, the experiment was started through addition of a mixture of 0.32 mg. of adenylyl polyphosphate P, 88  $\gamma$  of sulfanilamide, and fluoride to 0.05 M final concentration.

Experiment No.	Treatment of extract	Filtrate of boiled organ added, corresponding to gm. fresh weight	Sulfanilamide conjugated	Incubation time
1	Untreated Kept 16 hrs., 7°		$\gamma$	min.
			69	65
	Dialyzed 16 hrs., 7°	0.2 gm. rat liver	7	40
			58	
		0.2 gm. rat liver	0	65
		0.2 gm. rat liver	42	
2	Untreated Kept 16 hrs., 7-10°	0.4 " pigeon breast muscle	5	
		0.1 " dried yeast	0	
			59	50
			0	
	Kept 2 hrs., 37°	0.4 gm. rat liver	28	
		0.4 " pigeon breast muscle	14	
		0.004 M glutathione	0	
			26	
		0.4 gm. rat liver	48	
		0.4 " beef kidney	38	

*Comments*

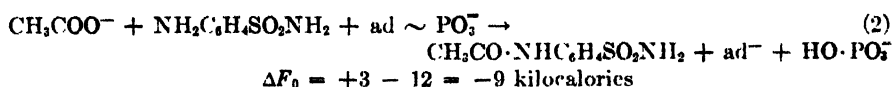
A coupling between respiration and acetylation of sulfanilamide, conforming with energetical considerations, had previously been found with liver slices (10) and is here confirmed with cell-free preparations. In chemical terms this observation signified the existence of a continuity of reactions between respiratory hydrogen transfer and acetate conjugation. Recent experiments (28) had shown that acetate may be phosphorylated in bacterial extracts through phosphate transfer from adenylyl pyrophosphate. In a first approximation, therefore, a linking between respiration and acetylation might be resolved into a sequence.





As a more remote possibility a primary phosphorylation of sulfanilamide might be considered.

The experiments reported in the second part of this paper support the general idea that the energy transfer between respiration and acetylation occurs by way of phosphate bonds. The present evidence, however, is largely in favor of assembling the separate steps sketched above into an inseparable reaction unit. The poor reactivity of acetyl phosphate makes a separate reaction between adenylyl pyrophosphate and acetate appear unlikely. It seems rather that acetate and sulfanilamide are enzymatically induced to form a complex with adenylyl pyrophosphate which exergonically breaks up into acetylsulfanilamide, adenylic acid, and inorganic phosphate.



The liberation of inorganic phosphate is overshadowed in the present conditions by the large phosphate liberation through adenylylpyrophosphatase. A stoichiometric participation of adenylyl pyrophosphate, however, in the process of acetylation is indicated strongly by the quantities required for acetylation. Furthermore, the endergonic nature of the condensation makes a coupling with an exergonic process necessary and excludes a mere catalysis. Although the total energy of the phosphate bond in adenylyl pyrophosphate  $\Delta F_0 = -12$  kilocalories is probably 3 to 4 times that required for the condensation ( $\Delta F_0 =$  about +3 kilocalories), an enzymatic mechanism is scarcely probable which would allow the energy derived from a breakup of a single bond to be utilized in multiple condensations.

Numerically the energy for this conjugation should be similar to that required for peptide synthesis, since the linkage formed in this reaction is a  $-\text{CO} \cdot \text{NH}-$  linkage. According to calculations by Borsook and Huffman (29), the synthesis of hippuric acid and of leucylglycine requires 2.5 and 2.9 kilocalories respectively. In spite of the aromatic nature of the amino group, such a comparison seems justified and may indicate applicability of the reaction scheme discussed here to peptide and protein synthesis.

#### SUMMARY

1. Homogenates of pigeon liver, when allowed to respire, acetylate considerable amounts of sulfanilamide; 2.5 ml. of homogenate of 1 gm. of liver under optimal conditions may conjugate up to 1 mg. per hour. Anaerobically the acetylation is slight, amounting to one-fifth or less of the aerobic capacity.

Acetylation may be found without the addition of metabolites, but

generally was doubled by the addition of acetate. Acetoacetate and pyruvate were only about half as active as acetate, and some increase was caused by the addition of acetoin.

2. Of a series of analogous compounds, sulfanilamide was most actively acetylated, followed by *p*-aminobenzoic acid, sulfathiazole, and sulfadiazine, in the indicated order.

In a few experiments with homogenates of livers from thiamine-deficient pigeons, the addition of cocarboxylase enhanced acetylation.

3. The supply of adenylyl pyrophosphate to the homogenate increased anaerobic acetylation to levels approaching the aerobic effect. The coupling with respiration could thus be replaced by a store of adenylyl pyrophosphate.

Experiments were undertaken to test acetyl phosphate for its capacity to furnish acetyl groups. These were complicated by the presence of a very active acetylphosphatase, an enzyme ubiquitous in animal tissues. Nevertheless, the results indicate rather definitely that acetyl phosphate is not active with the enzyme system. A non-enzymatic acetylation of aniline with acetyl phosphate could be demonstrated.

4. The enzymatic system, which brings about sulfanilamide acetylation through combination of the reactants with adenylyl pyrophosphate, is freely soluble and may be separated from particulate matter by centrifugation of the homogenate. Active solutions were obtained furthermore by extraction of acetone preparations of pigeon liver. Such enzyme preparations are easily inactivated by autolysis. Inactivation may be partly or wholly reversed through the addition of boiled liver juice.

5. The mechanism of acetylation by way of phosphate bond transfer is discussed.

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## OXYGEN POISONING\*

### III. THE EFFECT OF HIGH OXYGEN PRESSURES UPON THE METABOLISM OF BRAIN†

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The subject of poisoning by oxygen has been reviewed by the authors (1) and by Bean (3). Bert (4) first suggested that its cause may be the disturbance of some vital biochemical processes, particularly oxidative: consumption of oxygen, production of carbonic acid and urea, breaking down of glucose in the blood, all chemical phenomena which can be measured easily, appear to be considerably slowed down by the action of oxygen under high tension.

To study the phenomenon, the authors have determined the metabolic activity *in vitro* of surviving tissues exposed to high oxygen pressures in comparison with similar tissues under 1 atmosphere of oxygen. The present paper is concerned solely with experiments on brain tissue, which fall into three groups: (1) the intact animal was exposed, its symptoms observed, and certain aspects of the metabolism of its brain tissue subsequently studied; (2) the metabolism of the tissue was studied under normal circumstances after a preliminary exposure to high oxygen pressure; and (3) the metabolism was studied during exposure to high oxygen pressure in a specially built apparatus.

*Preparation of Tissues and Media:* In most of the experiments, organs from normal, fed albino rats were used. In all cases, the brains were removed immediately following decapitation of the animal, washed in saline, and rapidly prepared either by slicing or homogenizing. Homogenates were prepared from known weights of tissue in a glass homogenizer of the Potter-Elvehjem type with buffered medium in the ratio of 1:10, 15, or 20. Uniform slices of known thickness were prepared by means of a tissue microtome described elsewhere (5). This consists essentially of a Plexiglas holder in which a section of razor blade slides in such a way as to produce uniform slices with ease and rapidity. The extreme friability of brain slices makes

\* The first two papers of this series are "Oxygen poisoning: a review" (1) and "An apparatus for the determination of the gaseous metabolism of surviving tissues *in vitro* at high pressures of oxygen" (2).

† The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

blotting without injury difficult. Hence the determination of the initial wet weight was omitted. Instead the dry weight was determined at the end of the experiment as follows: To precipitate any proteins in solution in the medium, trichloroacetic acid was added to make a final concentration of 7 per cent. The contents of the Warburg respirator were then filtered through a weighed sintered crucible and the tissue on the filter washed three to four times with 1 to 2 ml. of water to remove salts and trichloroacetic acid. The dry weight was then determined after heating the crucible at 110° for 24 hours. This weight represented 100 per cent of the total solids in the original tissue, as demonstrated by the following control experiment.

Approximately 80 mg. (wet weight) of brain slices were equilibrated for 2 hours at 38° with 3 ml. of medium. 0.5 ml. of 50 per cent trichloroacetic acid was added and the mixture filtered. 3 ml. of the original medium without brain tissue were similarly treated. Both filtrates were analyzed for total solids, with the following result. The value calculated for the solids in the medium was 42.2 mg.; 42.2 mg. were observed in the medium after acidification and filtration, and 42.3 mg. after equilibration with brain, acidification, and filtration. It is evident that the solids in the filtrate from the brain slices were the same as those present in the original medium, proving that no solids present in the slices were lost during filtration or washing. Other controls showed that trichloroacetic acid was completely volatilized at 110°. It has been the custom in this laboratory to express metabolic activities on a wet weight basis in order to facilitate intercomparison of different organs. The wet weights were calculated from the dry weights, assuming a constant water content of 76.9 per cent which was the mean ( $\pm 0.3$  per cent) of fourteen control determinations on whole rat brains.

The equilibration media varied somewhat during the course of the experiments but consisted of a phosphate buffer (usually 0.040 M) at pH 7.2 (occasionally pH 6.7) with glucose (0.2 per cent). The osmolar concentration was 0.300 to 0.320 M. Besides sodium as chloride, it contained potassium (0.005 to 0.010 M), calcium (0.002 M), and magnesium (0.002 to 0.006 M). Variations from this medium are noted in the text when significant.

#### EXPERIMENTAL

*Intact Animal Experiments*—The animals were exposed to various pressures of oxygen in suitable pressure chambers. In the earlier experiments, a small chamber of about 600 ml. capacity was used, with a bottom layer of CO<sub>2</sub>-absorbing alkali (Shell Natron) and a Plexiglas top for observation. Control analyses of the chamber atmosphere showed less than 0.5 per cent CO<sub>2</sub> after 2 hours. In later experiments, the large, high pressure Warburg apparatus was used with Plexiglas side windows for the observation of symptoms. The gas capacity was about 25 liters; hence CO<sub>2</sub> accumulation

during the short experiments was insignificant. Compression of the animals by oxygen from standard cylinders was produced in 1 to 2½ minutes. Decompression was carried out in stages in some experiments, and rapidly in others. The brains were removed immediately following decompression. The prepared slices or homogenates were placed in standard Warburg vessels with appropriate medium, gassed (in the case of slices) with 100 per cent O<sub>2</sub>, and equilibrated at 38° with constant shaking. Oxygen uptake and CO<sub>2</sub> output were measured manometrically by the usual method.

*Preexposure Experiments*—The prepared slices or homogenate, in the appropriate medium, was placed in 20 ml. Erlenmeyer flasks mounted within a small pressure chamber which was then filled with oxygen at the required pressure and shaken in a water bath at 38°. The tissue was equilibrated at this pressure for the required time, decompressed rapidly, and transferred to a standard Warburg vessel for metabolic determinations.

*Experiments with Continuous Exposure to High Oxygen Pressure*—A special method (2) for the manometric determination of oxygen uptake and CO<sub>2</sub> output of tissues at high oxygen pressures was used. The apparatus consisted essentially of a cylindrical steel chamber of 100 liters capacity, which also serves as a water bath, equipped with windows and external controls permitting the operation of six specially adapted Warburg vessels and manometers. Temperature was maintained at 38° ± 0.05° and the vessels were shaken at the same speed (120 oscillations per minute) and with approximately the same amplitude as in the standard Warburg apparatus. The vessels were equipped with a center well containing a filter paper roll moistened with alkali for the absorption of CO<sub>2</sub>, and a chambered side stop-cock containing acid.

Immediately after preparation, the tissue was placed with appropriate medium in the main compartments of the vessels, which were gassed with 100 per cent O<sub>2</sub> and mounted in the apparatus. The chamber was closed and flushed for 7 minutes with O<sub>2</sub> at 15 pounds pressure (which was found sufficient to give an atmosphere of 98 to 99 per cent O<sub>2</sub>). The pressure was raised rapidly (8 atmospheres in 2 minutes), 8 minutes more being allowed to elapse for the physical solution of the O<sub>2</sub> and completion of temperature equilibrium. O<sub>2</sub> uptake was then measured manometrically. The respiratory period was terminated by killing the tissue with acid admitted from the chambered stop-cock. After sufficient time for the absorption of CO<sub>2</sub> liberated by the acid from the tissue and medium, the pressure within the chamber was lowered, and the vessels dismounted. The CO<sub>2</sub> absorbed upon the alkali-filter roll in the center well was determined in the usual way after transfer of the roll to a standard Warburg vessel. A parallel sample of tissue was acidified at the beginning of the respiratory period for the determination of the initial CO<sub>2</sub> of the system. Instead of acid for the deter-

mination of  $\text{CO}_2$ , auxiliary substances were at times admitted from the side stop-cock. In most cases, parallel slices from the same brain were equilibrated in the same medium in a standard Warburg vessel at 1 atmosphere of  $p\text{O}_2$ , and the respiration measured at the same time as those of the slices under high pressure.

In the studies on carbohydrate balance, the contents of the vessels were treated as follows: At the end of the respiratory period, tissue and medium were centrifuged, and the tissue washed with water before filtration through sintered crucibles for the determination of dry weights. Centrifugate and washings were brought to 10 ml. For lactic acid determination, 2 ml. were treated with 0.4 ml. of 50 per cent trichloroacetic acid, recentrifuged, washed, and filtered and the filtrate and washings were made up to definite volume and analyzed by the Barker-Summerson method (6). For glucose determination, to the remaining 8 ml. of the original centrifugate was added 0.5 ml. of 10 per cent  $\text{ZnSO}_4$  and  $\text{NaOH}$  until the solution gave a faint red color to phenolphthalein. After centrifugation, filtration, and washing of the residue with water, the filtrate and washings were brought to definite volume and glucose determined by the Benedict method.

The carbohydrate utilization during the respiratory period was calculated as follows: Parallel slices (usually two) were killed by the addition of acid at the beginning of the respiratory period and the glucose and lactic acid of their media determined. The means of these initial values were used to correct the final values obtained in the case of the respiring slices. Carbohydrate utilization was calculated as the decrease of glucose minus one-half of the increase of lactic acid and expressed as micromoles per gm.

*Standard Error of Means*—Where indicated this was calculated by Peter's formula as a sufficient approximation; *viz.*, standard error of the mean =  $1.25 \Sigma \text{a.d.} / (n \sqrt{n - 1})$ .  $n$  is the number of observations and  $\Sigma \text{a.d.}$  is the summation of the differences of the observations from the mean.

### Results

#### *Exposure of Intact Animal to High Oxygen Pressures (8 Atmospheres)*

Fig. 1 shows the data observed in eighteen animals exposed for 30 minutes to 8 atmospheres of oxygen. Section A shows the total incidence of central nervous symptoms. For simplicity they are grouped together as "convulsive symptoms" and include restlessness, twitching, clonic movements, tonic-clonic generalized convulsions, and spasms (most marked as opisthotonus occurring immediately after convulsions). The symptoms usually occur in this order and were each counted once when appearing in an animal; hence the incidence is greater than the number of animals. Dyspnea is also shown in Section A. The rôle of the central nervous system here is unknown; massive lung hemorrhages are almost always found following

exposure. Section B shows the incidence of complete collapse, a state from which the animals cannot be roused, and which usually ends in death. Section C shows the incidence of death. Six of the rats died during exposure. A few rats, obviously moribund, were sacrificed for pathological studies. Only an occasional rat recovered after decompression from the 30 minutes exposure which may, therefore, be considered between 90 to 100 per cent of the lethal dose.

In general, the most striking symptoms are of central nervous origin and are apparent from the 5th to the 25th minute of exposure to 8 atmospheres

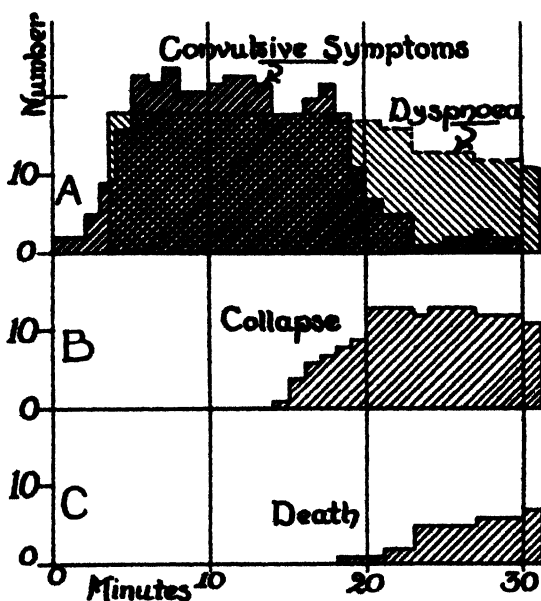


FIG. 1. Incidence of symptoms observed in eighteen albino rats exposed for 30 minutes to 8 atmospheres of oxygen. Six rats died during exposure and practically all died shortly after decompression.

of oxygen. They all suggest strong motor discharge. The significance of the constantly occurring dyspnea, and the associated lung pathology, do not concern the present study.

#### *Metabolic Studies*

*Brain of Normal, Fed Albino Rat;  $O_2$  Uptake and Respiratory Quotient*—It was found by control experiments with brain slices of different thicknesses that maximum respiration at 1 atmosphere of  $pO_2$  was achieved only with a maximum thickness of 0.35 mm., thicker slices giving significantly lower oxygen uptakes presumably owing to partial anaerobiosis in the center of



the slices. In all cases, the oxygen uptake was rectilinear for at least 2 hours; *i.e.*, the final rate was within 5 per cent of the initial rate. The control rates of oxygen uptake (see Table I) were significantly higher than most values cited in the literature. For example, Elliott and Libet (7) give a maximum initial rate corresponding to 149 micromoles per gm. per hour, but falling off with time. In earlier literature quoted by Quastel (8), similar or lower rates were found by Warburg, Loebel, and Dickens and Simer, while Quastel reported a rate similar to that obtained in the present study (197 micromoles per gm. per hour). We attribute our higher rates to the following factors: (1) improved slicing technique; (2) minimum trauma by the use of outside cortical slices with only one cut surface, and elimination of initial wet weight determination which requires blotting of the tissue. We emphasize that our results are based on true initial wet weights as demonstrated under "Methods."

TABLE I

*Mean Rates of Oxygen Uptake and Respiratory Quotients of Cortical Slices from Brains of Normal Fed Albino Rats, and Rats Exposed to 8 Atmospheres of Oxygen for 25 Minutes*

Rat treatment	O <sub>2</sub> uptake*	No. of determinations†	R.Q.	No. of determinations†
	<i>micromoles per gm. per hr.</i>			
Normal	186 ± 4.3	11	0.93 ± 0.008	7
High oxygen	190 ± 6	12	0.96 ± 0.005	12

\* All respirations were linear for 2 hours.

† Each determination on a separate rat.

The first category of experimental results included the O<sub>2</sub> uptake and R.Q. of brain slices following exposure of the normal, intact animal to 8 atmospheres of oxygen for 25 minutes.

The rates are compared to the mean normal values. There is no difference (Table I) in either rate of O<sub>2</sub> uptake or R.Q. resulting from exposure of the intact animal to high oxygen pressure sufficient to cause death in all cases. It is also significant that the respiration of these slices was rectilinear for 2 hours, and for 3 hours in six other experiments not included in Table I.

The rates (Table II) for brain homogenates from normal rats in 1 atmosphere of *p*O<sub>2</sub> were one-fourth those of the slices, and rectilinear for just over an hour, falling off slightly thereafter. It is possible that a number of factors such as the presence of Ca<sup>++</sup>, as mentioned by Elliott and Libet (7), are responsible for the low rates. It is not felt that this question is involved in the conclusions drawn in the present study, and that these con-

trol values shown are valid for comparisons with those at high oxygen pressure.

Table II shows the  $O_2$  uptake and R.Q. values of brain homogenates after exposure of the intact animals to 8 atmospheres of oxygen for 25 minutes. The only apparent effect upon the respiration by exposure of the animal is the shortening of the initial period of rectilinear respiration; *i.e.*, increase in the rate of falling off of oxygen uptake.

In preliminary experiments, brain slices or homogenates were exposed to high oxygen pressures for an initial period, and the effect determined by measuring the oxygen uptake in a final period at 1 atmosphere. In the case of slices, twelve determinations were made and the results were quite uniform. Following 1 hour's exposure to 6.9 atmospheres of oxygen, the subsequent oxygen uptake at the end of 1 hour averaged 68 per cent of that of control

TABLE II

*Mean Rates of Oxygen Uptake and Respiratory Quotients of Homogenates from Brains of Normal Fed Albino Rats, and Rats Exposed to 8 Atmospheres of Oxygen for 25 Minutes*

Each determination was made on a separate rat. Six determinations were made for each value reported. The total period of respiration for each determination was 90 minutes.

Rat treatment	Initial period of rectilinear respiration	Initial $O_2$ uptake	Final $O_2$ uptake	R.Q., total
	min	micromoles per gm. per hr.	micromoles per gm. per hr.	
Normal.	70	$46 \pm 1.5$	$35 \pm 1.2$	$0.87 \pm 0.020$
High oxygen	45	$49 \pm 1.7$	$32 \pm 1.9$	$0.81 \pm 0.010$

slices, falling to 52 per cent at the end of 2 hours. The mean respiratory quotient was  $0.94 \pm 0.03$ , not significantly different from that of the controls. Twenty-five measurements were made of the oxygen uptake of brain homogenates similarly treated. In general, the results were the same as with slices, although much more variable. Preliminary exposure ranging from  $\frac{1}{2}$  to 3 hours always resulted in decreases of subsequent oxygen uptake to a degree roughly proportional to the time of exposure and oxygen pressure. The  $O_2$  uptake in the final period varied from 20 to 80 per cent of that of the controls. The oxygen uptake was measured during the initial period at high oxygen pressures, as well as at 1 atmosphere in the final period. Fig. 2 shows data for a brain homogenate and is fairly typical of the behavior of homogenates under these conditions. Compared to the control observed throughout at 1 atmosphere, the initial rate of oxygen uptake at 8 atmospheres of oxygen fell off significantly during the 45 minutes of exposure. Following decompression to 1 atmosphere, the rate

continued to fall off, whereas the control sample of the same homogenate continued to take up oxygen in an essentially rectilinear manner.

A large number of observations were made of the oxygen uptake and R.Q. of brain slices during exposure to 8.0 atmospheres of oxygen, the high pressure apparatus being used for this purpose. In some cases the pressure was dropped to 1 atmosphere, and the observations continued. The results were sufficiently constant to be represented as means plus or minus the standard error of the mean (Table III). The times shown are those of exposure, although the readings were not begun until 8 minutes after high pressure was reached. Several striking findings are to be emphasized: (1) The initial rate of oxygen uptake, the tissue having been subjected to

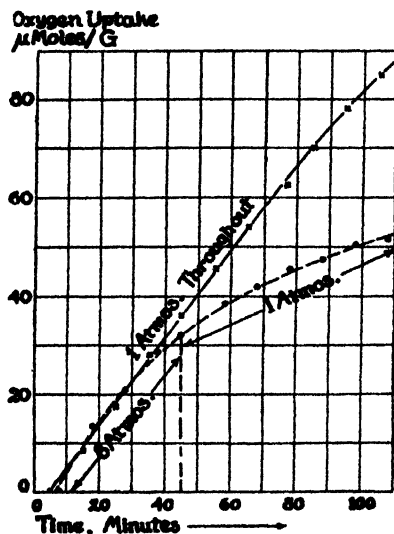


FIG. 2. The oxygen uptakes of homogenized rat brain, under 8 atmospheres of oxygen and subsequently at 1 atmosphere, and under 1 atmosphere throughout.

8 atmospheres approximately 8 minutes before the readings were begun, is not significantly different from that of the controls (Table I). (2) The oxygen uptake is well maintained for a period of 30 minutes, but reaches half its initial value in about 1 hour. The observed R.Q. values are less than those of the controls but whether this is significant or not cannot be stated. In the time required to kill an intact animal with severe manifestations of central nervous system disturbances (about 15 to 25 minutes) there is little or no change in the metabolism of the brain as indicated by the oxygen uptake and R.Q.

Figs. 3 and 4 demonstrate the significant fact that when oxygen poisoning is once initiated it is not reversible upon restoration of normal

conditions. In Fig. 3 the oxygen uptake was the same as that of the control during the 45 minute exposure to 8 atmospheres. But that poisoning has occurred is shown by the subsequent decrease of oxygen uptake at 1 atmosphere, until at 180 minutes the rate had fallen to 79 per cent of the control. In contrast, when the oxygen pressure is maintained at 8 atmospheres throughout (Fig. 4), the oxygen uptake falls off more rapidly after 60 minutes and is practically zero at 180 minutes. It is also to be noted that in comparison to the homogenates (Fig. 2), the slices are far more resistant to the poisonous action of high oxygen pressures.

To determine whether or not the effect of high oxygen pressure was a generalized one affecting the oxidation by brain of substrates other than

TABLE III

*Mean Rate of Oxygen Uptake and Respiratory Quotient of Cortical Slices of Brains from Normal Fed Albino Rats during Exposure of the Slices to High Oxygen Pressure*

	Time under pressure							R.Q.
	25 min	40 min	55 min	70 min.	100 min.	130 min.	155 min.	
Mean O <sub>2</sub> uptake, micromoles per gm per hr.	204 ± 7	185 ± 8	153 ± 10	108 ± 10	62 ± 7	36 ± 5	20	0.82 ± 0.028
% mean control	110 ± 4	99 ± 4	82 ± 5	58 ± 5	33 ± 4	19 ± 4	11	88 ± 3
No. of observations	18	18	13	10	6	3	1	10

glucose, experiments were done at 8 atmospheres of O<sub>2</sub> in the presence of various substrates.

The effects of oxygen poisoning were manifest with fructose, pyruvate, lactate, and succinate, as in all cases there was a falling off in oxygen uptake quite similar to that observed in the case of glucose. The oxidation of malate was not high even in the controls, but the rate of falling off was increased by high oxygen. Approximately the same held for succinate. Acetoacetate was used in an attempt to see whether a fatty acid intermediary reacted differently from carbohydrate intermediaries. However, the respiration in this case was so small relative to that of brain slices without any added substrates that no conclusion could be drawn. So far as this analysis has gone, no particular part of the carbohydrate oxidation system appears more affected by oxygen than another.

The possibility that the toxic action of oxygen is limited to the period when glucose is being metabolized was excluded by the following experi-

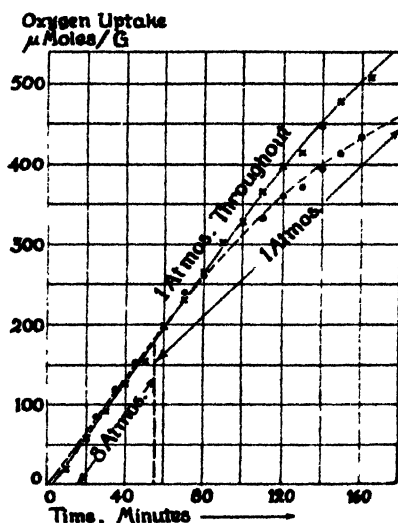


FIG. 3. The oxygen uptake of cortical rat brain slices, under 8 atmospheres of oxygen and subsequently at 1 atmosphere, and under 1 atmosphere throughout.

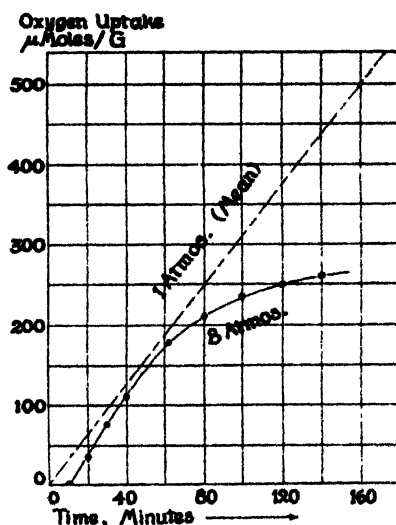


FIG. 4. The oxygen uptake of cortical rat brain slices under 8 atmospheres of oxygen compared with the mean of eleven observations at 1 atmosphere.

ments. Brain slices were equilibrated at 8 atmospheres of  $O_2$  with no added substrate, or with pyruvate. After 60 minutes exposure glucose was added.

In either case the oxygen uptake fell off from the beginning and the addition of glucose at 60 minutes produced no effect except a slight transient increase in the sample without substrate.

The possibility that some specific action of oxygen upon the pyruvate-oxidizing system could be prevented or diminished by the presence of large excesses of thiamine was tested. Brain slices with pyruvate plus additional thiamine did not differ from the controls without thiamine in their reaction to high oxygen pressure. Nor did the late addition of thiamine produce any significant effect. This experiment failed to indicate any specific effect of high oxygen pressure on the pyruvate oxidation system.

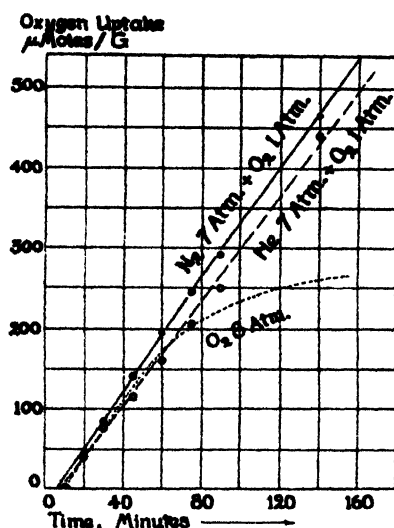


FIG. 5. The oxygen uptake of cortical rat brain slices under high nitrogen or helium pressures compared with that under 8 atmospheres of oxygen.

As a further control the effects of high pressures of nitrogen or helium (together with 1 atmosphere of oxygen) on the oxygen uptake of brain slices were studied. Either nitrogen or helium under pressure is used in deep sea diving operations and the former is known to have narcotic action upon the intact organism, while helium, at similar or greater pressures, is known to be inert.

In comparison with a typical curve for tissue slices under 8 atmospheres of oxygen (Fig. 5), it is apparent that under 7 atmospheres of nitrogen or helium (and 1 atmosphere of oxygen) the oxygen uptake was rectilinear for 140 minutes and the same in magnitude as with 1 atmosphere of oxygen alone. This evidence shows that the phenomena observed under high oxygen pressure are specific and not due to simple mechanical pressure.

The metabolism of the brain as determined by *in vivo* and *in vitro* experiments is mainly carbohydrate in character.

Experiments were done to determine whether or not high oxygen pressure qualitatively alters this character. Brain slices were equilibrated, and the glucose utilization determined as described under "Methods." In addition the oxygen uptake was observed. The data in Table IV show the following. (1) The net glucose utilization is not significantly diminished at high pressures. (2) The ratio of oxygen uptake to glucose utilization is not signifi-

TABLE IV

*Effect of Oxygen at 8.2 Atmospheres on Carbohydrate Metabolism of Brain Slices from Normal Fed Albino Rats*

The carbohydrate balance and oxygen uptake are expressed in micromoles per gm. (80 minutes).

Pressure of oxygen	Rat No.	Change of glucose	Change of lactic acid	Glucose balance	O <sub>2</sub> uptake	Ratio, oxygen uptake to glucose balance
<i>atmospheres</i>						
1	94D-14	-43	+36	-25	180	7.2
		-37	+33	-20	179	9.0
1	94D-17	-42	+47	-19	120	6.3
		-48	+72	-12	150	12.5
1.7	94D-18	-73	+61	-43	252	5.9
		-58	+41	-38	246	6.5
Mean $\pm$ standard error of mean...		-50 $\pm$ 8	48 $\pm$ 7	-26 $\pm$ 5	188 $\pm$ 20	7.9 $\pm$ 1.1
8.2	94D-13	(-45)*	(+47)*	(-22)*	(114)*	5.2
		-63	+70	-28	138	4.9
	94D-16	-60	+78	-21	156	7.4
		-78	+107	-25	168	6.7
Mean $\pm$ standard error of mean. . .		-67 $\pm$ 5	85 $\pm$ 8	-24 $\pm$ 2	154 $\pm$ 10	6.0 $\pm$ 0.7

\* The first determination was for 47 minutes; it is excluded from the mean. Duplicate determinations were made on each rat.

cantly different from 6.0, the expected value if the chief metabolism is that of carbohydrate. High oxygen pressure produced no change in this relationship. (3) There appears to be more lactic acid formation (glycolysis of glucose) at high pressure. The data are insufficient to state that this is a constant manifestation of oxygen poisoning. The main conclusion drawn is that high oxygen, while diminishing total metabolism as shown by decrease of oxygen uptake, has no specific effect upon the ability of the tissue to oxidize glucose which it continues to do at a diminished rate.

The possible enhancement of the toxic action of high pressures of oxygen by partial pressures of  $\text{CO}_2$  at and above normal levels and hence associated with normal or low pH is widely discussed in the literature. Experiments were therefore done which differed from those previously described in that tissues were studied in 8 atmospheres of oxygen containing various partial pressures of  $\text{CO}_2$ . Direct determinations of oxygen uptake cannot be done in the high pressure Warburg apparatus when there is  $\text{CO}_2$  in the gas phase. We therefore measured the  $\text{CO}_2$  output. For this purpose parallel slices of brain were assembled in two identical systems. Initially both contained

TABLE V

*Carbon Dioxide Output of Cortical Slices from Brains of Normal Fed Albino Rats in Presence of  $\text{CO}_2$  and Oxygen at 1 or 8 Atmospheres*

Experiment No.	$\text{pO}_2$ atmospheres	Final pH	Final $\text{pCO}_2$ mm. Hg	$\text{CO}_2$ output micromoles per gm. per hr.
1	1	7.3	39	143
2	1	7.3	46	117
3	1	7.1	68	114
4	1	6.9	44	125
5	1	6.7	72	110
6	1	6.5	126	57
7	1	6.1	151	87
8	8	6.6	107	145
9	8	6.8	40	234
10	8	6.8	72	59
11	8	6.5	38	104
12	8	6.5	36	124
13	8	6.9	37	83
14	8	6.9	37	127
15	8	6.9	43	99
16	8	6.9	40	98
17	8	6.9	43	73
18	8	7.0	30	93

the same amount of  $\text{CO}_2$  in both solution and gas phase as determined in control experiments. One set was killed at zero minute by the introduction of strong alkali, and the other at the end of the respiratory period (1 hour). By customary methods the total  $\text{CO}_2$ , which was completely absorbed by the alkali, was determined in each, the difference being the  $\text{CO}_2$  output for the period. The medium was similar in all respects to the phosphate medium used in the experiments with oxygen alone except that the phosphate was reduced to 0.005 M and bicarbonate was added to make the concentration either 0.010 or 0.020 M. The data are presented in Table V and for comparison similar data obtained at 1 atmosphere of oxygen are



included. The results are in accord with our experience with high oxygen in the absence of  $\text{CO}_2$ ; *i.e.*, the  $\text{CO}_2$  output (and hence the oxygen uptake) was only slightly affected by high pressures of oxygen +  $\text{CO}_2$  during the 1 hour's exposure. There is no indication that the presence of  $\text{CO}_2$  over a considerable range of pressure resulting in a wide range of pH enhanced the toxic action of the oxygen.

#### DISCUSSION

Oxygen at 8 atmospheres produces definite toxic effects quickly in rats, culminating in convulsions within 15 minutes. Death uniformly occurs if the exposure is continued 15 minutes more. Obviously, the processes resulting in death are very rapid. Up to a certain stage, however, they are reversible because, even after convulsions, many animals recover completely following decompression. The facts and implications must be borne in mind when the result of *in vitro* study of tissues under high oxygen pressure is examined.

Gross autopsy findings were confined to hemorrhagic patches of intestines and lungs. However, it cannot be stated that important pathological changes do not occur in the brain under high oxygen pressure, since detailed histological studies have not been completed.

The insignificant reduction in the rate of oxygen uptake of brain slices from animals killed by high oxygen pressure is difficult to reconcile with the severe symptoms and death of the animal. Several explanations are conceivable: (1) Some toxic substance is formed by the action of high oxygen pressure *per se*, the precursor being some normal metabolite. (2) Some essential factor is oxidized by oxygen *per se*, the oxidized product being deprived of its essential action. In these two alternatives enzyme systems do not enter. (3) An enzyme system is poisoned in such a way that its normal function is disarranged so as to produce a toxic substance, presumably from some normal metabolite. (4) Some enzymatic system producing a factor essential to normal coordination of central nervous function is peculiarly susceptible to high oxygen pressure. In consequence it is rapidly inhibited, the essential factor is not produced, and the train of symptoms and death result.

It is impossible from present knowledge to decide among these alternatives. Preliminary experiments in which blood from animals killed by high oxygen pressure was injected into normal rats failed to support the first or third hypothesis. However, the possibility that the hypothetical toxic substance might rapidly revert to a non-toxic form must be borne in mind, since our experiments did not eliminate this possibility. Some color is given to the fourth alternative by the fact that some enzymes are com-

pletely resistant to high oxygen pressures, others are moderately affected, while some are quickly and completely inhibited. If the hypothetical enzyme concerned is an oxidative one, then its oxygen requirements for normal function are such a small part of the total metabolism of the brain that its elimination escapes detection when the over-all metabolism is measured by the Warburg method. The possibility that the inhibited enzyme is quickly reversed to a normal status upon return to 1 atmosphere of oxygen (a necessity of our experimental technique) must be considered. In that event, the subsequent oxygen uptake at 1 atmosphere would be normal. Against this is our failure to restore the oxygen uptake of inhibited brain slices or homogenates by removal to 1 atmosphere. The nature of the hypothetical system or its metabolic product is entirely a matter of speculation. However, it must be remembered that the animals with severe symptoms under high oxygen pressure may be completely restored to normal, if decompressed early enough, indicating a possible quick reversal of an inhibited enzyme system to normal.

The possibility that oxygen poisoning is a tissue anoxia (the so called hyperoxic anoxia) has been discussed by Bean (3). Several mechanisms are conceivable to explain this anoxia: (1) an inactivation by high oxygen pressures of oxidative enzymatic systems; (2) loss of the dual function of hemoglobin. As previously discussed (1) the blood is thereby slightly modified from the normal state; *i.e.*, the pH is 0.02 to 0.04 lower, and the partial pressure of the blood  $\text{CO}_2$  is 4 to 8 mm. of Hg higher. These circumstances are assumed to be peculiarly favorable for the toxic action of oxygen; (3) creation at capillary walls by some mechanism not known of an impermeable barrier so that, despite a high partial pressure of oxygen in the capillary blood, oxygen cannot diffuse into the cells.

We have no evidence on the last of these alternatives, but our experiments are clearly against the first two. When brain tissue from normal animals was exposed to high oxygen pressures, there was no rapid reduction in oxygen uptake. Nor was there any rapid change in the respiratory quotient or decrease in the oxidation of carbohydrate. We emphasize strongly that the reduction in oxygen uptake which was observed, in comparison to the symptoms of the intact animal, developed slowly. In fact *it was not apparent to a measurable degree in the time required to produce violent convulsions, collapse, and death in the animal.* Decrease of oxygen uptake under high oxygen pressure of tissues other than brain, as will be shown in the accompanying paper, develops even more slowly. In other words, the observed metabolic changes are *subacute* as distinguished from the *acute* general poisoning. Our experiments on the intact animal killed by high oxygen pressure showed that the brain tissue, when subsequently examined

at 1 atmosphere of oxygen, was not different from controls with respect to oxygen uptake.

It has been suggested that there is a considerable accumulation of  $\text{CO}_2$  during oxygen poisoning, although the best evidence indicates that it is small (1). Bean (3) has discussed the possibility that the resultant increased acidity of the tissues and particularly the increased  $p\text{CO}_2$  have some specific rôle in enhancing the toxic action of oxygen. The simultaneous administration to the intact animal of  $\text{CO}_2$  with high oxygen pressure might hasten the onset of the acute phase by vasodilatation which would result in quicker saturation of the tissues with oxygen. But that increased  $\text{CO}_2$  and acidity combine with oxygen to produce an unusual degree of tissue anoxia cannot be supported on the basis of our experiments, for we have shown that increase of both factors considerably beyond that observed in animals poisoned with oxygen does not cause early development of oxygen uptake of brain tissue, nor does the later decrease occur more rapidly than with oxygen alone.

We have been unable to obtain any evidence that the acute phase of oxygen poisoning characterized by convulsions, collapse, and death is associated with a rapidly developing inhibition of oxidative enzymes of the tissues. Therefore we find it difficult to suppose that tissue anoxia in the intact animal is the cause of the symptoms. So far as carbohydrate metabolism is concerned, our experiments are inconclusive in determining whether or not any particular part of the oxidizing cycle is more sensitive to high oxygen pressure than any other. The oxidation of fructose, pyruvate, and lactate all showed approximately the same general type of change as that found with glucose; *i.e.*, a relatively slow decrease. Perhaps the oxidation of pyruvate is more quickly inhibited, but more data are needed for a final decision. No conclusions can be drawn from the experiments on malate and succinate, because these substrates were not metabolized actively, and the methods employed were not sensitive enough to detect any changes under pressure. The same applies to the one intermediary product of fat metabolism studied, acetoacetic acid.

Attempts to reverse the subacute process on brain tissue were unsuccessful. This is in partial distinction to the acute effects on the intact animal, for in many cases complete recovery followed exposure even after the occurrence of severe convulsions. Following exposure of the tissue, simple reduction of pressure to 1 atmosphere of oxygen did not restore the oxygen uptake to normal. On the contrary, the process once initiated appeared to continue, as shown by the progressive fall in the rate of oxygen uptake. The meaning of this is not apparent. The addition of fresh glucose after exposure had no effect, nor did the presence of a considerable excess of thiamine prevent the inhibition of pyruvate oxidation.

## SUMMARY

1. Toxic effects developed rapidly in rats exposed to 8 atmospheres of oxygen and culminated in convulsions. Practically all animals exposed for 30 minutes died, either during exposure or within a few hours after decompression.

2. Up to a certain point, oxygen poisoning was completely reversible, for if decompressed relatively early even after severe convulsions many animals recovered.

3. Gross autopsy findings were hemorrhagic patches in the lungs and intestines. Gross examination of the brain showed no changes.

4. Thin slices of brain from animals killed by high oxygen pressure showed no significant changes in total oxygen uptake or R.Q. when these were subsequently determined by the Warburg technique at 1 atmosphere of oxygen.

5. Similar preparations of brain slices and homogenates from normal animals were exposed in a preliminary period to high oxygen pressures and subsequently observed in the Warburg apparatus at 1 atmosphere of oxygen. There was found a steadily decreasing rate of oxygen uptake in proportion to the pressure of oxygen and the time of exposure. The inhibition of brain metabolism by high oxygen was similar with glucose, fructose, pyruvate, or lactate as substrate.

6. Similar findings were obtained when normal tissue was observed at 8 atmospheres of oxygen. The initial rate of oxygen uptake was halved in approximately 1 hour. The time required for appreciable reduction in rate of oxygen uptake was far greater than for the production of serious symptoms, convulsions, and death when intact animals are subjected to the same oxygen pressure.

7. Brain slices subjected to relatively high pressures of  $\text{CO}_2$  (up to 107 mm. of Hg) in addition to 8 atmospheres of oxygen were no more susceptible to oxygen poisoning than those exposed to 8 atmospheres of oxygen alone.

8. The metabolism of brain *in vitro* although diminished was not altered from its predominantly carbohydrate character by high oxygen pressures.

9. Attempts to reverse the lowered oxygen uptake of brain tissue poisoned by high oxygen pressures by early return to 1 atmosphere failed.

10. The rate of oxygen uptake of brain tissue *in vitro* at 1 atmosphere of oxygen was not affected by 7 additional atmospheres of nitrogen or helium.

11. On the basis of our findings the division of oxygen poisoning into an acute and subacute phase is discussed. It was concluded that generalized tissue anoxia is not the cause of acute oxygen poisoning.

12. To explain the marked discrepancy between the apparently normal metabolic function of brain and the severe symptoms and death of animals in the *acute* phase of oxygen poisoning, alternative hypotheses are discussed.

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## OXYGEN POISONING

### IV. THE EFFECT OF HIGH OXYGEN PRESSURES UPON THE METABOLISM OF LIVER, KIDNEY, LUNG, AND MUSCLE TISSUE\*

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Since the time of Bert the possibility that the poisonous action of high pressures of oxygen is due to the inhibitory action of oxygen upon the oxidative enzyme systems of tissues has been extensively discussed (1, 2). The number of direct observations in the literature of the metabolism of tissue under high oxygen pressure is, however, quite limited. We have studied the subject by determining the oxygen uptake of tissue preparations *in vitro* by the Warburg technique. By use of an apparatus previously described (3) it is possible to measure the oxygen uptake and  $\text{CO}_2$  output of tissues when they are equilibrated with 8 atmospheres of oxygen. In Paper III (4) data from experiments with rat brain have been presented. In this paper we present further data of similar character on isolated surviving liver, kidney, lung, and muscle tissue of the rat.

The technique of the preparation of tissues, equilibration, etc., are fully discussed elsewhere (4).

*Liver*—In a series of eighteen control determinations of the oxygen uptake of liver slices from normal, fed white rats a mean value of  $70 \pm 4$  micromoles per gm. per hour over a period of time from 0.5 to 5.5 hours was found. The mean r.q. (thirteen determinations) was  $0.72 \pm 0.024$ . An important fact to be noted is that the oxygen uptake did not depart significantly from rectilinearity for 5.5 hours. When the oxygen uptake of liver slices was measured during exposure to oxygen pressures from 5.0 to 8.4 atmospheres, the results recorded in Table I were found.

For better intercomparison the results are reported as percentages of the rate of oxygen uptake of slices from the same liver at 1 atmosphere of oxygen. Two points are established by inspection of the mean of the series: (1) The rate during the 1st hour did not differ significantly from that at 1 atmosphere. (2) As in the case of the brain, the rate of oxygen uptake fell slowly and steadily during exposure. However, the rate has reached 50 per cent of the control rate only after 3.5 hours, as opposed to 1 hour in

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

the case of the brain. After 3.5 hours the fall is slower. The respiratory quotient is not significantly different from that of the controls.

TABLE I

*Oxygen Uptake and Respiratory Quotients of Liver Slices from Normal Fed Wistar Rats during Exposure of Slices to High Oxygen Pressure*

The oxygen uptakes are expressed as percentages of the uptake of parallel slices from the same livers observed simultaneously at 1.0 atmosphere of  $pO_2$ , except as indicated. Zero time is taken as the moment  $O_2$  pressure reached a maximum.

Experi- ment No.	pO <sub>2</sub>	Exposure time								R.Q.	
		1.0 hr.	1.5 hrs.	2.0 hrs.	2.5 hrs.	3.0 hrs.	3.5 hrs.	4.0 hrs.	5.0 hrs.		6.0 hrs.
		Oxygen uptake, per cent of controls									
	atmos- pheres										
94A-1	5.0	133	119	106	100	98	83	72	52	31	
94A-3	6.2	85	68	84	78						
94A-2	6.4	149	149	149	149						
94A-4	6.6	94	64	41	30	23					
		129	114	85	68	53					
94A-5	6.8	127	108	83							
		100	67	58	45	33	25				
94A-7	7.2	129									0.85
		129	100	70							0.80
		108	108	87	69	45					0.92
94A-6	7.3	103	103	103	71						0.48
		103	103	103	64	57	50	43			
94A-9	8.0	103									
		114									
		106									
		123									
96-3	8.3	136									0.50
94A-8	8.4	160									
		118	118								0.96
		120	100	75							0.81
		143	143	125	98						0.84
		116	116	91	80	66	57	48			0.70
Mean.....		119	105	90	77	54	54	54	52	31	0.76
± standard error of mean.....		4	6	7	8	9	12	10			0.06

Some of the rates of oxygen uptake during the 1st hour are higher than those of the control series. However, we do not believe that this signifies a real increase in metabolic oxygen, but was due to a technical error which was eliminated in later experiments. It was found (3) that excessive

amounts of rubber connections in the apparatus took up in physical solution appreciable amounts of oxygen at high pressure. When this source of error was eliminated by glass to glass connections, the oxygen was the same as that under 1 atmosphere (Experiment 94A-9).

This experience was universal with all tissues studied; we have never been able, when the above source of error was minimized or eliminated, to demonstrate conclusively any significant increase of oxygen uptake during the initial period of observation under high oxygen pressures. If such occurs, it must be less than 10 to 15 per cent of the total oxygen uptake; otherwise it would have been detected. However, it requires 2 minutes to raise the pressure in the apparatus and readings cannot be made until physical solution of oxygen in the medium is complete (approximately 8 minutes more); hence the possibility that there is a significant increase of oxygen uptake during this time cannot be rigorously excluded. If such an increase occurs, it must be of short duration so that it does not affect the rate 10 to 15 minutes from the time the maximum pressure is reached. The significance of the point lies in the possibility that reactions absent or minimum at 1 atmosphere of oxygen might be greatly accelerated under high oxygen pressures and be concerned with oxygen poisoning.

In general, the same comment made for the brain slices can be made here: though there is a definite effect by high oxygen pressures on the oxygen uptake of liver slices, its extent is negligible until long after the time required to extinguish the last signs of life in the intact animal under similar conditions.

*Kidney*—In preliminary experiments kidney slices from rats were exposed to high oxygen pressure, and then the oxygen uptake measured with oxygen at 1 atmosphere. In general relatively long preliminary exposures were required to reduce the oxygen uptake significantly. With oxygen at 7 atmospheres, exposure for 1, 2, and 3 hours resulted in a subsequent oxygen uptake, compared to controls maintained throughout at 1 atmosphere, of 70, 50, and 35 per cent respectively.

When the oxygen uptake of kidney slices was measured during exposure to high oxygen pressures, the data shown in Table II were obtained. With the exception of one experiment (No. 94C-11) the results are reasonably consistent. In general, the sensitivity of the kidney slices to high oxygen lies midway between that of brain and liver, and again it is seen that the falling off in respiration takes place chiefly long after the intact animal would have succumbed. The mean R.Q. value is not significantly different from that observed at 1 atmosphere.

*Lungs*—Mongrel dogs, were used after prolonged exposure to 1 atmosphere of oxygen.

It is well known that animals exposed for long periods to 1 atmosphere



of oxygen develop marked pulmonary symptoms and die as a result. The marked edema and patchy hemorrhage found in such animals have been described by many observers since the first reports of Bert. On account of their obvious interest we studied the metabolism of slices from the lungs of five dogs which had been kept in a chamber containing 0.8 to 1.0 atmos-

TABLE II

*Oxygen Uptake and Respiratory Quotients of Kidney Slices from Normal Fed Wistar Rats during Exposure of Slices to High Oxygen Pressure*

The oxygen uptakes are expressed as percentages of the uptake of parallel slices from the same kidneys observed simultaneously at 1.0 atmosphere of  $pO_2$  except as indicated. Zero time is taken as the moment  $O_2$  pressure reached a maximum.

Experiment No.	$pO_2$	Exposure time							R.Q.
		0.5 hr.	1.0 hr.	1.5 hrs.	2.0 hrs.	2.5 hrs.	3.0 hrs.	4.0 hrs.	
	<i>atmospheres</i>								
94C-1	6.4	151	151	151					
94C-2	5.2	100	88	72	50	32	20		
94C-3	5.6	100	87	65	56	48	33	15	
94C-4	5.8	115	69	50					
		143	84	63	50	38	29		
94C-5	5.3		103	94	80				
94C-6	6.0	103	103	59	44	32	24		0.63
94C-7	7.2	84	84	42	32	23	16		0.61
		84	84	42					0.54
94C-8	8.0	139	117	69	57	42			
		116	104	69	60				
94C-9	7.7	127	127						0.53
		131	131	102	82				0.68
		131	131	102	82	61	51		0.65
94C-10	8.6	121	74	64					0.79
94C-11	8.0	41	31						
		40	29						
		65	48						
		61	45						
Total mean $\pm$ standard error of mean...		103 $\pm$ 8	90 $\pm$ 8	75 $\pm$ 8	59 $\pm$ 6	39 $\pm$ 4	29 $\pm$ 3	15	

phere of oxygen from 48 to 116 hours, and which were sacrificed before death.<sup>1</sup> In all cases the oxygen uptake of the slices was determined at 1 atmosphere of oxygen.

All results are expressed on a dry weight basis to avoid error in com-

<sup>1</sup> We are indebted to Dr. John Lockwood of the Harrison Department of Research Surgery, University of Pennsylvania, who supplied and treated these animals.

parison with normal animals, since the variable amount of lung edema in the experimental animals precluded comparison on a wet weight basis. Nineteen determinations on lung slices from normal mongrel dogs gave a mean oxygen uptake of  $131 \pm 3$  micromoles per gm. of dry weight per hour. The mean R.Q. was  $0.83 \pm 0.02$ . The data on dogs exposed to oxygen are

TABLE III

*Oxygen Uptake and Respiratory Quotients of Slices of Lung from Mongrel Dogs Exposed to Moderate Pressure of Oxygen*

Dog No.	Oxygen	Exposure	Dry weight to wet weight ratio	O <sub>2</sub> uptake	R.Q.
	<i>atmosphere</i>	<i>hrs.</i>	<i>per cent of control</i>	<i>micromoles per dry gm. per hr.</i>	
917	0.8	48	82	159	0.86
	0.8	48	66	125	0.63
921	0.8	48	76	108	0.73
926	0.8	48	83	128	0.72
619	1.0	116	78	181	0.82
Mean $\pm$ standard error of mean.				$140 \pm 15$	$0.75 \pm 0.04$

TABLE IV

*Oxygen Uptake and Respiratory Quotients at 1 Atmosphere of Oxygen of Slices of Lung from Normal Fed White Rats following Exposure of Slices to High Oxygen Pressures*

Oxygen pressure, preliminary period	Exposure time	Subsequent oxygen uptake	Per cent of control rate	R.Q.
<i>atmospheres</i>	<i>hrs.</i>	<i>micromoles per dry gm. per hr.</i>		
2.2	2	271	114	
		269	109	
4.2	1	292	99	
		212	73	
4.2	2	207	82	
		231	93	
6.9	1	217	93	
		233	81	
6.9	2	170	61	0.82
		167	55	0.87

given in Table III. In all cases, the lungs showed the typical patchy hemorrhage and edema, the latter being reflected in a decreased dry weight to wet weight ratio. However, on the average, there was found no significant change in the oxygen uptake or the R.Q. On the basis of these experiments it is possible to exclude any specific effect of prolonged exposures

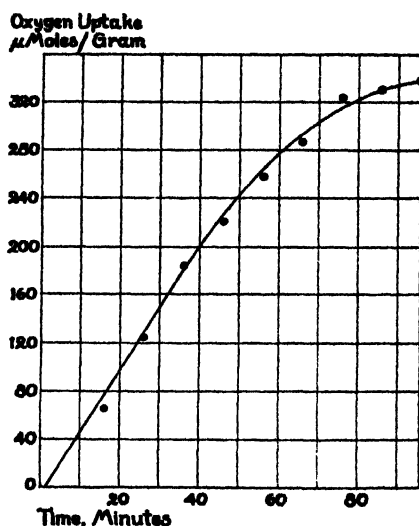


FIG. 1. Oxygen uptake (micromoles per dry gm.) of slices of lung from normal white rat during exposure to 8 atmospheres of oxygen.

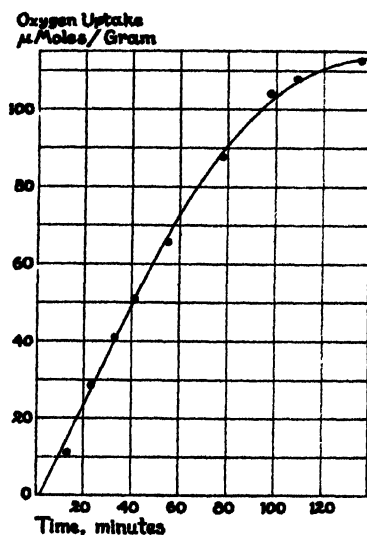


FIG. 2. Oxygen uptake (micromoles per wet gm.) of diaphragm from white rat during exposure to 8 atmospheres of oxygen.

to oxygen at pressures approximating 1 atmosphere causing significant decreases of the metabolic activity of pulmonary tissue.

White rats were used in twenty-two control determinations on the oxygen uptake of slices of lungs; the mean value was  $289 \pm 3$  micromoles per dry gm. per hour and the r.q. was  $0.94 \pm 0.01$ . The oxygen uptake (corresponding to a  $Q_{O_2}$  of 6.5) is surprisingly high, considering the histological character of the lung, which would lead one to suppose that the organs contain little metabolically active tissue. The high r.q. also indicates a predominantly carbohydrate type of metabolism. In a preliminary series lung slices were exposed to high oxygen pressures for 1 or 2 hours, the oxygen uptake being subsequently measured at 1 atmosphere. The data (Table IV) show, as with other tissues, that exposure up to 1 hour at 6.9 atmospheres has relatively little effect upon the subsequent metabolism. Nor is the r.q. significantly affected. In experiments in which the oxygen uptake was measured during exposure to high oxygen pressure essentially the same results were obtained. Fig. 1 shows the course of oxygen uptake of rat lung slices at 8 atmospheres of oxygen. As repeatedly observed with other organs, the rate of oxygen uptake remains unchanged for a period of time approximating 1 hour, after which it falls off slowly, reaching half the initial rate in about 2 hours.

*Muscle*--The oxygen uptake of striated muscle (rat diaphragm) was measured during exposure to 8 atmospheres of oxygen (Fig. 2). Muscle is more resistant to oxygen poisoning than all other tissues examined. The initial rate under high oxygen pressure is not significantly different from that under 1 atmosphere. It falls off slowly, reaching half the initial rate in about 3 hours.

#### DISCUSSION

The tissues of the rat which have been examined are slowly poisoned by high pressures of oxygen. In the course of a relatively long time, considering the high pressures used, the oxygen uptake begins to fall off and eventually ceases. The organs may be arranged roughly in a descending order of susceptibility to high oxygen pressure; *viz.*, brain, kidney, liver, lung, muscle. We have repeatedly made the point, however, that the loss of metabolic activity develops slowly and for that reason we have concluded that it plays little or no rôle in the acute phase of oxygen poisoning or the early death of intact experimental animals. Supportive evidence for this conclusion is found in our experiments on dogs exposed for long periods of time to 1 atmosphere of oxygen. Despite the marked pulmonary changes which in comparable animals leads eventually to death, we observed no significant change of the metabolic activity of lung slices. The significance of these findings to the problem of oxygen poisoning has been fully discussed in Paper III (4).

## SUMMARY

1. The effect of high oxygen pressures on the metabolism of slices of liver, kidney, lung, and muscle was determined.
2. The relation of these findings to the problem of oxygen poisoning is discussed.

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# THE INDOPHENOL-XYLENE EXTRACTION METHOD FOR ASCORBIC ACID AND MODIFICATIONS FOR INTERFERING SUBSTANCES\*

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There is no lack of methods for the determination of ascorbic acid, but none gives any great assurance of specificity. The indophenol reduction methods are probably the simplest, and these have been checked with animal assays for a number of fresh and cooked vegetables. The most generally adopted indophenol method has been that of Bessey (1), a method in which the excess dye is measured by photoelectric means, thereby offering a greater degree of objectivity than with the titration procedure. Provisions are made for correction of color and turbidity in the tissue filtrates and for substances which reduce the dye more slowly than ascorbic acid. With certain products, particularly fruits, the color of the blank may be several times that of the dye, leading to inaccuracies in the color measurements. From a practical standpoint, the need of two photoelectric measurements for each determination, and the time and care required for immediate readings and observation of possible "drifts" due to other reducing substances, are excessive when large numbers of determinations must be made. Some of these disadvantages have been eliminated by use of the principle described by Bukatsch (2) who noted that the excess oxidized dye may be extracted with xylene, and methods employing this principle have been described by Stotz (3) and Pepkowitz (4).

None of these methods, however, offers the desired specificity in the presence of a variety of other reducing substances which may occur in various heated, stored, and processed foods. Such non-ascorbic acid reducing substances include stannous and ferrous salts, sulfite, sulfhydryl compounds, reductic acid, and the reductones. Some of these, particularly the reductones, are most difficult to distinguish from ascorbic acid. Levy (5) has proposed the use of peroxide to eliminate sulfite interference. To account for reductones, Mapson (6) and Snow and Zilva (7) have devised methods which in principle involve condensation of the ascorbic acid, but not of the reductones, with formaldehyde. The use of the dinitrophenylhydrazine procedure of Roe and Kuether (8) and Roe and Oesterling (9), although eliminating some of the reducing materials, introduces other types of interfering substances.

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In this paper, an improved indophenol-xylene extraction method is described, and peroxide and formaldehyde modifications are presented which minimize the interference due to reduced tin and iron, sulfite, and reductiones. This increase in specificity has been made possible without unduly complicating the routine usefulness of the method. Applications of the method to selected food products are given, and the extent of interference by thiol and quinol compounds is recorded. (The basic method described and its modifications have been found satisfactory and convenient in this laboratory for the past year in the routine analysis of foods, particularly highly colored products, and those containing significant proportions of non-ascorbic acid reducing materials. We have noted sufficient instances of food products containing large proportions of non-ascorbic acid reducing substances so that we now consider it essential to apply regularly the procedures described, especially with food products which have been subjected to long heat treatments or extensive storage, before accepting values obtained by an unmodified indophenol method.

### Procedure

#### Reagents—

*Acetate buffer*, pH 4.0. Mix 1 liter of 50 per cent sodium acetate ( $\text{NaAc} \cdot 3\text{H}_2\text{O}$ ) and 1 liter of glacial acetic acid.

*Dye*. Dissolve 25 mg. of 2,6-dichlorobenzenone indophenol in 200 ml. of warm distilled water, and filter. The solution keeps in the refrigerator for at least 2 weeks.

*Xylene*. Unless specifically tested for suitability, this should be redistilled (see "Testing of method"). (Eastman Kodak technical grade has been found satisfactory if distilled.) Xylene used in the method may be recovered by being shaken with a 20 per cent solution of sodium hydroxide to neutralize acetic acid, and redistillation.

*Peroxide*, 3 per cent. Dilute 30 per cent C.P. hydrogen peroxide 10-fold. Prepare weekly.

\* *Formaldehyde*, technical grade, 40 per cent solution.

*Hydroquinone*. Add an equal volume of acetone to a saturated solution of hydroquinone in acetone, to obtain a half saturated solution. This must be prepared fresh before use.

The specific methods described apply to extracts of tissues prepared with fresh 2 per cent metaphosphoric acid in 1 N sulfuric acid. When other types of acid extractions are employed, the analyst should adjust the dilution of the acetate buffer to obtain a final pH of 3.6 to 3.8 in the basic method and its modifications.

With each new product to be analyzed, it should be determined whether the basic method (Procedure A) may be employed, either without or with a hydroquinone blank, or whether the peroxide modification (Procedure

B) or formaldehyde modification (Procedure C) should be used. To determine whether the acid filtrate contains pigments which are extracted by xylene, the basic method (Procedure A) is applied to the filtrate without the addition of dye, and the xylene extract examined for color. Filtration of the extract through a fairly retentive paper (Whatman No. 2) usually removes the finely suspended particles containing carotene which occur with some products, and which otherwise result in slightly yellow xylene extracts. We have thus far encountered xylene-soluble pigments only in the filtrates of certain food products subjected to long heating, which then required the hydroquinone blank described under Procedure A. In such cases the quantity of pigment extracted depended on the time of shaking; hence the hydroquinone decoloration procedure was considered more accurate than to employ a blank without the dye, as suggested by Pepkowitz (4).

■ If the product is known to contain sulfite as a preservative, or to contain iron or tin from long storage in cans, the peroxide modification (Procedure B) may be routinely used with little extra trouble. Possible interference by tin or iron can be detected by a comparison of the results of applying Procedures A and B on the same sample. A similar comparison of results with the basic method (Procedure A) and the formaldehyde modification (Procedure C) will determine whether reductones are present in appreciable amounts. The latter may occur commonly in various processed and stored food products (see Table II), but much less frequently in fresh products.

Proof of the applicability and limitations of these methods, with mixtures of ascorbic acid and the various interfering materials, is given under "Testing of method."

*Basic Method, Procedure A*—A 1 to 5 ml. sample of acid filtrate, containing 0.02 to 0.15 mg. of ascorbic acid, is placed in a 150 × 18 mm. test-tube. An equal volume of acetate buffer is added (this is sufficient to adjust 6 per cent  $\text{HPO}_3$  or 2 per cent  $\text{HPO}_3$  in 1 N  $\text{H}_2\text{SO}_4$  to pH 3.6 to 3.8), followed immediately by 2 ml. of dye and complete mixing; 10 ml. of xylene are at once added, a cork is inserted, and the tube is shaken vigorously for 6 to 10 seconds to extract the excess dye. A second tube ("total dye") to determine the amount of dye added is prepared by the same treatment of a volume of extracting medium equivalent to the sample employed in the determination. A standard tube is likewise prepared which contains 0.100 mg. of ascorbic acid in the same volume of extracting medium. Upon completion of a series, the tubes are centrifuged briefly to separate the layers sharply, and the top xylene layers are poured off or withdrawn and measured in a suitable photoelectric instrument at 500  $\text{m}\mu$  or with a filter which transmits maximally at this wave-length. Xylene solvent is used as a reference sample.

Since ascorbic acid may be quite unstable after addition of buffer of



pH 4.0, and excessively long contact with the dye permits the reaction of slower reducing substances to become appreciable, it is essential to add buffer, dye, and xylene in rapid succession. The time required for complete interaction of ascorbic acid and dye elapses during the mixing of reactants and addition of xylene. In a series of determinations, the operation upon each tube is therefore completed through the xylene stage before proceeding with the successive sample of acid filtrate.

If preliminary tests show that xylene removes color from the acetate-buffered tissue extract, then an additional operation is required with the sample tube. After the first reading, 2 drops of hydroquinone (in acetone) are added to the xylene extract and mixed. The orange color due to the excess dye is bleached within 30 seconds, after which a second reading is taken to measure the blank due to the tissue extract. If the xylene layers are transferred to a single cuvette for the photoelectric measurements, it is advisable to read a complete series, then to return the xylene layers to the original tubes, and to add hydroquinone to the whole series before reading the tissue blanks. This eliminates the possibility of hydroquinone from one sample contaminating a successive one containing dye in the xylene layer.

*Peroxide Modification, Procedure B*—To eliminate the reducing action of sulfite or reduced iron and tin, peroxide is added prior to the dye. For this purpose, a volume of 3 per cent hydrogen peroxide equal to the acetate-buffered extract, or a smaller volume of more concentrated peroxide sufficient to result in a 1.5 per cent solution, is added just before the dye. The remainder of the determination follows as in the basic method. Total dye and standard tubes are prepared as previously described, containing a total volume before dye addition equivalent to that of the sample tube.

*Formaldehyde Modification, Procedure C*—To correct for the action of reductones, the reducing action of ascorbic acid is selectively abolished by interaction with formaldehyde at pH 3.6 to 3.8 for 10 minutes. In practice, two tubes are required for the determination. The first, Tube 1, contains the selected volume of tissue filtrate; the second, Tube 2, containing the same volume of filtrate, receives an equal volume of acetate buffer, followed by a half volume of 40 per cent formaldehyde (e.g. 2 ml. of sample, 2 ml. of buffer, 1 ml. of formaldehyde) to give an 8 per cent formaldehyde solution. The tubes are allowed to stand at room temperature for 10 minutes. At the end of this period, Tube 1 receives its volume of acetate buffer and a half volume of water to yield a volume equivalent to that of Tube 2. 2 ml. of dye are then added to each tube, mixed, and the excess dye extracted with 10 ml. of xylene. The difference between the amounts of dye reduced in the two tubes represents the true ascorbic acid.

Neither peroxide nor formaldehyde affects the stability of the dye in the xylene layer.

### Calculations

*Dye Equivalent*—The amount of dye equivalent to a standard amount of ascorbic acid must be determined for the particular instrument and colorimeter tubes employed, and should be checked frequently. Amounts of dye present in the xylene extract are conveniently expressed as the  $\log I_0/I$  (absorption) at 500 m $\mu$ . Should the instrument invariably be set at 100 ( $I_0$ ) with xylene solvent, then  $\log I_0/I$  may also be expressed as 2 minus  $\log I$ , where  $I$  is the per cent transmission of the sample tube. The dye equivalent of ascorbic acid may be calculated from the total dye and standard tubes as follows:

$$0.1 \text{ mg. ascorbic acid} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{standard}}$$

$$0.1 \text{ mg. ascorbic acid} = \Delta \log_{\text{standard}}$$

$\Delta \log_{\text{standard}}$  ranges from 0.290 to 0.330 with the instruments employed in this laboratory.

*For Basic Method and Peroxide Modification*—The dye used by the ascorbic acid in the sample is

$$(1) \quad \Delta \log_{\text{sample}} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{sample}}$$

The amount of ascorbic acid in the sample tube is then

$$(2) \quad 0.1 \text{ mg.} \times \frac{\Delta \log_{\text{sample}}}{\Delta \log_{\text{standard}}}$$

Provided  $I_0$  is always 100, a simple graph may be utilized by plotting per cent transmission (on a logarithmic scale) against mg. of ascorbic acid (on a linear scale). A straight line is drawn between the points 0 ascorbic acid-per cent transmission of the total dye tube and 0.1 mg. of ascorbic acid-per cent transmission of the standard tube. Transmissions of sample tubes may then be converted to ascorbic acid values directly from the graph.

If hydroquinone is employed in the basic method, then  $\log I_0/I_{\text{sample}}$  in Equation 1 must be replaced by

$$\log I_0/I_{\text{sample}} - \log I_0/I_{\text{hydroquinone bleached}}$$

*For Formaldehyde Modification*—

$$(3) \quad \Delta \log_{\text{total reducing substances}} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{Tube 1}}$$

$$(4) \quad \Delta \log_{\text{reductions}} = \log I_0/I_{\text{total dye}} - \log I_0/I_{\text{Tube 2}}$$

$$(5) \quad \Delta \log_{\text{ascorbic acid}} = \log I_0/I_{\text{Tube 2}} - \log I_0/I_{\text{Tube 1}}$$

From each  $\Delta \log$  value, the mg. of material (expressed as ascorbic acid) can be calculated by use of Equation 2.

### *Testing of Method*

*Basic Method*—The principle of the basic method, namely the complete extraction of the oxidized dye by xylene, has been adequately tested by Bukatsch (2), Stotz (3), and Pepkowitz (4). Nelson and Somers<sup>1</sup> noted that the color of the xylene layer may increase on standing (dye oxidation) with some samples of solvent. It is therefore important either to test the suitability of the sample of xylene employed, or to redistil routinely the xylene as recommended in this paper. The finding of a linear relation between the log of absorption of the dye in xylene and the amount of ascorbic acid has been repeatedly confirmed in this laboratory. The use of the basic method permits the analysis of extracts of such highly colored food materials as beet, cherry, raspberry, and grape, without resort to expensive and time-consuming electrometric methods.

*Peroxide Modification*—Sulfite is able to reduce the dye under the conditions of the test, and this may occur in sulfite-treated foods in amounts which affect the accuracy of the method. In the peroxide modification of the titrimetric procedure employed by Levy (5), an error may arise owing to oxidation of the reduced dye by the peroxide. The end-point may vary according to the speed of the titration. The peroxide technique is particularly adapted to the xylene method, owing to the short contact of the dye with peroxide.

Reduced tin or iron also acts like ascorbic acid in dye reduction methods, and may occur in canned products, particularly acid products stored for considerable periods. Some of the increases in ascorbic acid noted on storage in cans may actually arise from the contamination with iron or tin.

Table I illustrates the interference caused by iron, tin, and sulfite, and the correction effected by the simple peroxide modification.

*Formaldehyde Modification*—Some fresh products, but particularly products which have undergone extensive heat treatment or long storage, may contain substances collectively termed reductones. It has been difficult to distinguish these from ascorbic acid, even by enzymatic means. Although these substances have not been identified chemically, it seems probable that they resemble the glucose-amine type of compound rather than the reductones produced by the action of alkali on sugars or the reductive acid formed in the reaction of pentoses and strong acid (10).

Mapson (6) has observed that formaldehyde condenses with ascorbic acid to form a complex that no longer reduces the indophenol dye. Snow

<sup>1</sup> Nelson, W. L., and Somers, G. F., personal communication.

and Zilva (7) have made a careful study of the rate of formaldehyde condensation with varying concentrations. Their experiments showed that at pH 3.5, with an 8 per cent formaldehyde concentration, the ascorbic acid was almost entirely combined, while reductones and reductic acid were only 15 and 5 per cent combined, respectively. These methods involve procedures not readily amenable to routine analysis. Our study of the formaldehyde reaction with regard to time and pH led to the simple adaptation recommended in this paper.

As shown in Table II, a significant proportion of the reductones, reductic acid, and glucose-amine complex, artificially prepared, still acts like ascorbic acid in the formaldehyde modification. On the other hand, the naturally occurring reductones of stored dehydrated cabbage appear to be more sharply distinguished from ascorbic acid in their relation with formaldehyde,

TABLE I  
*Interference Caused by Reduced Iron, Tin, and Sulfite, and Its Correction by Peroxide (Procedure B)*

0.10 mg. of ascorbic acid in each tube.

Interfering material	Molar ratio of material to ascorbic acid	Procedure A, "ascorbic acid"	Procedure B, ascorbic acid
		mg	mg
None		0.099	0.100
Fe <sup>++</sup>	1:1	0.138	0.099
	1:1	0.121	0.101
Sn <sup>++</sup>	1:1	0.104	0.100
	20:1	0.117	0.098
SO <sub>3</sub> <sup>-</sup>	10:1	0.114	0.100
	25:1	0.134	0.098
	100:1	0.205	0.099

one sample of which showed no combination with formaldehyde. Until the exact nature of the non-ascorbic acid reducing materials is recognized in different products, it cannot be claimed that the formaldehyde modification described offers an exact analytical discrimination between these materials and ascorbic acid. Nevertheless the procedure provides a closer approximation of the true vitamin value, and has provided us with a much greater assurance that our analyses represent true ascorbic acid.

Table II also illustrates the great discrepancies that may result from the conventional dye reduction analysis of several food products, and the more nearly correct values obtained by the formaldehyde modification. It is advised that food products be first tested by the formaldehyde modification to determine the possible presence of significant amounts of non-ascorbic acid reducing materials before adopting a simple dye titration or the basic method of this paper.

*Other Interfering Substances*—There remain other substances which may be encountered in the analysis of foods for which no adequate corrections are available, or for which specialized methods are necessary. Cysteine and reduced glutathione, at an equimolar concentration with ascorbic acid, increase the ascorbic acid result by 7 to 9 per cent, and at a 10:1 molar concentration by 40 to 50 per cent. These sulfhydryl compounds, like ascorbic acid, condense with formaldehyde at pH 3.6 to 3.8, but, if known to be present in interfering concentrations, can be eliminated by formaldehyde condensation at pH 0.6 (6). At the latter pH, these compounds

TABLE II

*Interference Caused by Reductones and Related Compounds, Correction by Formaldehyde (Procedure C), and Illustrative Applications*

Material	Apparent ascorbic acid (no formaldehyde)	Ascorbic acid (with formaldehyde modification)
	mg. per 100 gm.	mg. per 100 gm.
Ascorbic acid	5.0	5.0
Synthetic reductone solution (11)*	24 0	8.4
Reductic acid solution (12)	1.9	0.30
Glucosamine complex†	3.2	0 35
Dehydrated cabbage, light color	139	131
“ “ dark “	71	0
“ “ “ “	60	4.2
Blackberry jelly	6.2	1 8
Guava-mix “	20.6	14.6
Apple-raspberry juice, 0° storage.	1 1	0
“ “ 37° “	2 8	0 17
Apple juice	1.1	0 18
Grapefruit juice	28 1	28 0
Sauerkraut “	16.4	13.5

\* The figures in parentheses represent bibliographic reference numbers.

† Prepared by warm storage of a solution containing glucose, glycine, phosphate, and malic acid.

condense with formaldehyde; ascorbic acid does not. Thiourea, addition of which has been suggested to prevent darkening of fruits (13), does not interfere at a molar ratio of 10:1.

In the commonly used hydrogen sulfide reduction method for dehydro-ascorbic acid, it is known that the quinols and sulfhydryl compounds formed may interfere with the dye reduction method. As quinol types we have tested catechol, tannic acid, pyrogallol, and hydroquinone. Catechol, at a 100:1 molar ratio with ascorbic acid, and tannic acid, at 10:1, cause insignificant errors; pyrogallol and hydroquinone, at 10:1, cause 25 to 50 per cent increases in the ascorbic acid values. Unfortunately their

rate of reaction with the dye depends on the amount of ascorbic acid present. No suitable means have been found to correct for the error due to these substances.

#### SUMMARY

1. A rapid, accurate indophenol-xylene extraction method for the determination of ascorbic acid in common and highly colored food products has been described.

2. Interference by artificially prepared reductone and reductic acid has been largely eliminated. Normally occurring non-ascorbic acid reducing material, at least that in dehydrated cabbage, appeared to be completely differentiated from ascorbic acid by formaldehyde.

3. The extent of interference from sulphydryl and quinol compounds is discussed.

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# THE LIPID PARTITION OF ISOLATED CELL NUCLEI OF DOG AND RAT LIVERS\*

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Lipids are essential cellular components and in combination with proteins are basic materials in the architecture of the cell (2). They are known to be especially important in the structure of cell membranes (3) and are present in such cytoplasmic constituents as the mitochondria and sub-microscopic particulate lipoprotein complexes (4). Their presence in the cell nucleus, although long in doubt, has been established recently by analysis of isolated cell nuclei (5-7).

The nuclear lipids have special significance in view of the indications (5) that their functional rôle is structural rather than metabolic. With the availability of a suitable method for separating the nucleus from the cell cytoplasm and the establishment of lipids as nuclear components it became possible to investigate the differential distribution of the lipids present in the cell nucleus.

## EXPERIMENTAL

Cell nuclei were isolated by the method of Dounce (6) from the livers of normal dogs, normal rats, and rats fed an experimental diet containing butter yellow (*p*-dimethylaminoazobenzene). This isolation procedure consists essentially in rapidly adding the sample (100 to 150 gm.) of frozen liver to a Waring blender containing cracked ice in distilled water plus citric acid to adjust the pH to between 6.0 and 6.2. After blending to break the cell membranes, the material is strained through cheese-cloth and the intact cell nuclei isolated by differential centrifugation and sedimentation. The purity of the nuclear preparations was checked by microscopic examination. After isolation, the samples of cell nuclei were dried from the frozen state under a vacuum (cryochem). Comparable samples of the whole liver tissue were also dried by the same method (8).

The dried material was extracted with hot ethanol, followed by ethyl ether. The final extract, combined, represented approximately a 3:1

\* Read by title before the American Institute of Nutrition at the annual meeting scheduled for Cleveland, May 8-10, 1945 (1).



mixture of alcohol-ether and was analyzed for total phosphorus, choline, sphingomyelin, total and free cholesterol, galactose, and acetone-soluble glycerol, by previously published methods (9). From these analyses, total phospholipid, choline phospholipid, cephalin, lecithin, cholesterol esters, cerebrosides, and neutral fat were calculated (10).

The lipid composition of the preparations of isolated cell nuclei and of comparable samples of whole liver tissue are given in Table I. The distribution pattern of the lipid components is given in Table II.

*Dog Liver*—The main differences between the lipid composition of the nuclei and that of the whole liver tissue are in the neutral fat and phospholipid portions. The nuclei contain 4.6 per cent (dry weight basis) neutral fat in comparison with 6.92 per cent in the whole cell. In contrast,

TABLE I

*Lipid Composition of Whole Liver Tissue and Liver Cell Nuclei*

The values are in per cent of dry weight.

	Dog liver, normal		Rat liver			
	Whole tissue	Nuclei	Normal		Tumorous	
			Whole tissue	Nuclei	Whole tissue	Nuclei
Total lipid	17.18	16.54	15.22	18.13	22.73	14.67
Neutral fat.	6.92	4.60	4.13	4.18	11.66	4.41
Essential lipid.	10.26	11.94	11.09	13.95	11.07	10.26
Cerebroside	0	0	0.33	0	0.72	0.29
Free cholesterol	0.12	0.18	0.37	0.36	1.14	0.64
Cholesterol esters.	0.95	1.02	2.01	1.14	1.15	2.04
Phospholipid	9.19	10.74	8.38	12.45	8.06	7.29
Cephalin	3.47	2.87	2.15	3.38	3.34	2.28
Lecithin	5.29	7.37	5.80	8.71	4.23	4.43
Sphingomyelin	0.43	0.50	0.43	0.36	0.49	0.58

the nuclei contain a larger concentration of phospholipid (10.74 per cent) than the whole liver tissue (9.19 per cent). Likewise, the essential lipid (10) (total lipid minus the variable or storage lipid fraction, neutral fat) is higher in the nuclei.

The essential lipid fraction, which is comprised of those lipids important in cellular structure, phospholipid and cholesterol, makes up approximately three-fourths of the total lipid of the nucleus (72 per cent). Furthermore, 90 per cent of the essential lipid is phospholipid, of which over two-thirds is the lecithin type and less than 5 per cent is the sphingomyelin type of phospholipid. No cerebroside was detected in the dog liver.

*Normal Rat Liver*—The results on the normal rat liver show even more striking differences in the lipid composition of the nuclei and the whole

liver cell than were found for the dog liver. The nucleus contains approximately 14 per cent essential lipid (dry basis) as compared to 11.09 per cent for the whole liver tissue. As noted for dog nuclei, 90 per cent of the essential lipid content of the rat liver nuclei is phospholipid, of which 70 per cent is lecithin and only 3 per cent is sphingomyelin. Apparently, the neutral fat is about equally distributed, since both the whole tissue and the nuclei contained 4 per cent of this component. Likewise, free cholesterol occurs in similar concentration in both the cell cytoplasm and nucleus. However, the nuclei contained only half as much (1.14 per cent) cholesterol ester as did the whole liver tissue (2.01 per cent). Although

TABLE II  
*Percentage Lipid Distribution*

	Dog liver, normal		Rat liver			
			Normal		Tumorous	
	Whole tissue	Nuclei	Whole tissue	Nuclei	Whole tissue	Nuclei
Per cent of total lipid						
Neutral fat	40	28	27	23	51	30
Essential lipid	60	72	73	77	49	70
Per cent of essential lipid						
Cerebroside	0	0	3.0	0	6.5	2.8
Free cholesterol	1.2	1.5	3.3	2.6	10.3	6.2
Cholesterol esters	9.2	8.5	18.1	8.2	10.4	19.9
Phospholipid	89.6	90.0	75.6	89.2	72.8	71.1
Per cent of total phospholipid						
Cephalin	37.7	26.7	25.7	27.1	41.4	31.3
Lecithin	57.6	68.6	69.2	70.0	52.5	60.8
Sphingomyelin	4.7	4.7	5.1	2.9	6.1	7.9

the rat liver contained a small amount of cerebroside (0.33 per cent), none could be detected in the nuclei.

*Tumorous Rat Liver*—The whole liver tissue from rats with tumors (hepatomas and adenocarcinomas (11)) produced by feeding *p*-dimethylaminoazobenzene showed a much greater total lipid concentration than did the nuclei isolated from the same tissue. This elevated total lipid is largely the result of an increased concentration of neutral fat in the tumorous liver. The nuclei contained 4.41 per cent neutral fat, which is only slightly higher than that found in normal rat liver nuclei (4.18 per cent). The tumorous whole liver tissue, however, contained 11.66 per cent neutral fat.

The tumorous liver cell nuclei showed marked alterations in essential lipid content and the distribution of the components comprising this par-

ticular fraction. Not only the concentration of the essential lipid but that of both the phospholipid and free cholesterol was less concentrated in the nuclei than in the whole cell. On the other hand, cholesterol esters were twice as concentrated in the nuclei (2.04 per cent) as in the whole tissue (1.15 per cent). The whole tumorous liver tissue contained a relatively high content of cerebroside (0.72 per cent) and, in contrast to the normal cell nuclei in which no cerebroside was detected, the tumorous liver cell nuclei contained 0.29 per cent cerebroside on the dry weight basis. As a result of these marked changes in concentration, the essential or structural lipid pattern of the nuclei was greatly altered from that of the normal. Approximately 20 per cent of the essential lipid was cholesterol esters, whereas only 71.1 per cent was phospholipid.

#### DISCUSSION

It seems apparent that, in general, the lipid pattern of the normal liver cell nucleus is similar to that of whole liver tissue. This is particularly true of the dog liver preparations. The significant differences appear to be quantitative rather than qualitative.

The nuclei from the normal livers of both the dog and rat have significantly greater amounts of phospholipid than the whole liver cell. In fact, 90 per cent of the essential lipid of the nucleus is phospholipid. Furthermore, in contrast to the composition reported for other types of cell nuclei (muscle, muscle tumor, and pus (5)), the major portion of the phospholipid is lecithin and cephalin (95 to 97 per cent), the lecithin fraction alone comprising 70 per cent.

In view of the supposedly structural rôle of the lipids in the nucleus it is surprising to find such a high content of the metabolic type, neutral fat, present. The nucleus contains between 4 and 5 per cent neutral fat and although the whole rat liver tissue contains a similar proportion of triglyceride, that in the nuclei is not merely a reflection of the whole tissue, as is shown by the results on the dog and tumorous livers. The dog liver tissue had nearly 7 per cent neutral fat and the tumorous liver, 11.7 per cent.

This study gives evidence of the profound changes a pathological condition produces in cellular structure, reflected by alterations in the lipid pattern, and confirms previous investigations of a similar nature (2). The striking changes shown by the tumorous cell nuclei are the greatly reduced concentration of phospholipid and the increased amount of cholesterol ester. Both alterations indicate tissue degeneration, according to the work of Bloor and his school (2, 12, 13). This is also in line with the observations that the enzyme-synthesizing activity of neoplasm chromosomes is reduced (14) and that the activity of most enzymes found in hepatomas induced by butter yellow is less than that of normal liver tissue (15).

One further notable change from normal in the lipid composition of abnormal nuclei is the presence of cerebrosides in the tumorous cell nuclei. It is difficult to assess the significance of this until further studies are completed.

Although the total lipid content of the tumorous rat liver is over one-third higher than normal, one is impressed with the similar amounts of essential lipid, 11.09 in the normal and 11.07 per cent in the tumorous tissue. This, together with other relationships (the higher concentration of phospholipid in normal nuclei than in whole tissue, the relatively low concentration of phospholipid in the abnormal nuclei, the slightly lower concentration of phospholipid in the whole tumorous liver than in the normal liver, and the fact that most of the lipid present is phospholipid) has raised the question of whether the pathological changes, particularly with respect to phospholipid, occur primarily in the nucleus. It cannot be answered directly until it is possible, experimentally, to isolate cell nuclei quantitatively from the whole tissue. However, a rough estimation can be attempted at this time, based on the data of Marshak (16), who determined the ratio between the volume of the liver cell nucleus and total cell volume by microscopic measurement and found it to be approximately 6:100.

Assuming, therefore, that 6 per cent of the liver is nuclei, from Table I, 100 gm. of rat liver contain 8.38 gm. of phospholipid, and 6 gm. of nuclei. 6 gm. of nuclei (12.45 per cent phospholipid) would contain 0.75 gm. of phospholipid; therefore,  $8.38 - 0.75 = 7.63$  gm. of phospholipid present in the remainder of the cell (94 gm.) and  $(7.63/94) \times 100 = 8.12$  per cent phospholipid present in the cell cytoplasm.

A similar calculation for the tumorous rat liver yields a value of 8.11 per cent phospholipid for the tumorous cell cytoplasm.

If the above assumption, based on the measured ratio between the volumes of nucleus and cell, is valid, the calculations indicate that the pathological derangement in the phospholipid content of the liver cell, produced by the administration of *p*-dimethylaminoazobenzene (butter yellow), occurs in the nuclei.

#### SUMMARY

The lipid (phospholipid (cephalin, lecithin, and sphingomyelin), free and combined cholesterol, cerebroside, and neutral fat) distribution was determined in cell nuclei isolated from the livers of normal dogs, normal rats, and rats fed butter yellow (*p*-dimethylaminoazobenzene), and in comparable samples of the whole liver tissue.

Although the lipid pattern of the liver cell nucleus is qualitatively similar to that of the whole liver tissue, it appears to be significantly different quantitatively. The essential or structural type of lipids comprise 12 to 14

per cent of the dry weight of the nucleus, or approximately three-quarters of the total lipid content. 90 per cent of the essential lipid is phospholipid of which less than 5 per cent is sphingomyelin. The pathological condition which occurs in the liver after feeding butter yellow apparently produces a significant change in the structure of the liver cell, indicated by alterations in the lipid pattern. The nuclei isolated from the tumorous liver cells exhibit a greatly reduced concentration of phospholipid and an increased amount of cholesterol ester. Calculations indicated that the pathological (butter yellow tumor) derangement of the phospholipid content of the liver cell occurs primarily in the nucleus.

The implications of these results from one pathological anomaly suggest a need for studies on other types of conditions involving cellular structure and function.

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# THE EFFECTS OF ANOXIA AND OF INJECTED CYTOCHROME *c* ON THE CONTENT OF EASILY HYDROLYZABLE PHOSPHORUS IN RAT ORGANS\*

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It is now well established that the easily hydrolyzable phosphorus fraction, particularly adenylyl pyrophosphate, plays an important rôle in the tissue transfer of energy (1). This compound continuously donates phosphoric acid radicals to other metabolites, and hence requires continuous resynthesis. For this resynthesis molecular oxygen is essential. One might therefore anticipate that under conditions of anoxia this resynthesis would be impaired, and hence the content of easily hydrolyzable phosphorus would be diminished. The chain of events resulting from such inhibited resynthesis of easily hydrolyzable phosphorus might contribute considerably to the general pathological consequences of tissue anoxia.

It was our purpose to determine whether a diminution in the content of easily hydrolyzable phosphorus could actually be demonstrated to result from anoxia and, if so, what might be done to overcome this effect of low oxygen tension.

In the experiments reported below it is demonstrated that anoxia produces a sharp decrease in the content of easily hydrolyzable phosphorus in rat organs. We had earlier found that some of the effects of anoxia on the intact organism could be prevented by the previous administration of cytochrome *c* (2). We therefore attempted to determine whether cytochrome *c* would likewise prevent this decrease in the content of easily hydrolyzable phosphorus.

## *Methods*

The following methods were employed. The easily hydrolyzable fraction was determined according to the procedure of Lohmann (3). The organs of decapitated animals were weighed on the analytical balance immediately after death, transferred to a small beaker containing 5 cc. of ice-cold 5 per cent trichloroacetic acid, coarsely minced with scissors, and then homogenized. The suspension was returned to the beaker and any particles adher-

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† We wish to acknowledge the technical assistance of Mrs. Bella Wadler.

ing to the walls of the homogenizer were transferred to the beaker by rinsing with 5 cc. of 5 per cent trichloroacetic acid. In convenient aliquots of the clear trichloroacetic acid filtrate, phosphorus determinations were carried out according to the method of Fiske and Subbarow (4) directly and after 7 and 30 minutes hydrolysis in *N* sulfuric acid.

According to Lohmann (3), these values after hydrolysis for 7 minutes represent the content of the easily hydrolyzable phosphorus of the adenylyl pyrophosphates. Kerr (5) recommended a hydrolysis time of 15 minutes for this purpose. The assumption that the figures represent adenylyl pyrophosphate is based largely on studies dealing with striated muscle. In

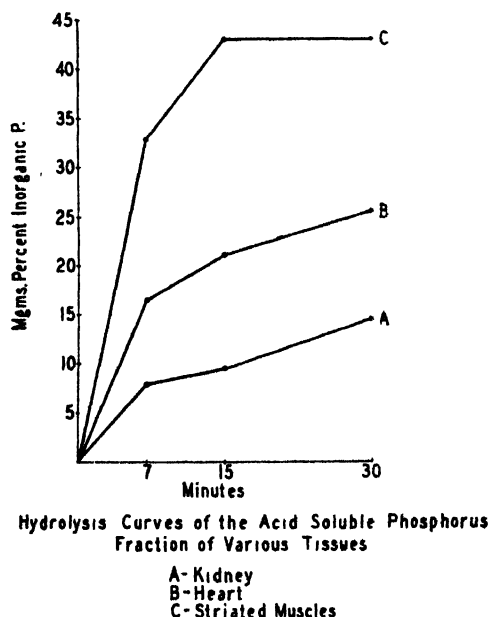


FIG. 1

order to check the validity of this interpretation as related to heart muscle and kidney tissue, we determined hydrolysis curves on these two organs. These curves showed a definite break between 7 (or 15) and 30 minutes, as shown in Fig. 1. Therefore, one may conclude that the error caused by the hydrolysis of other phosphoric esters is of a similar order of magnitude to that in striated muscle. On this basis we have assumed that the phosphorus values in our experiments recorded below represent largely the easily hydrolyzable phosphorus of adenylyl pyrophosphate. The samples of cytochrome *c* used for the injections were prepared from beef heart according to the method of Keilin and Hartree (6).

*Treatment of Animals*—In all experiments on anoxic animals, there arises the problem of properly defining the condition of anoxia. Anoxia is a biological phenomenon; hence, the effective threshold of the oxygen concentration and the time over which it is applied will vary in individual animals.

In order to overcome these difficulties, the experiments were carried out on groups of rats obtained on the same day from the same source. All rats of such a group were of the same age and weight, although not always from one litter. They were raised and maintained under the same conditions.

When an experiment was carried out, a group of rats was anesthetized with ether, and one-half of the number of animals was injected intravenously with 5 mg. of cytochrome *c*. 10 minutes after the injection, all animals were placed in a chamber in which an atmosphere of 3 per cent oxygen and 97 per cent nitrogen was maintained by a continuous flow of the gas mixture. Since we had found in preliminary experiments that uninjected rats usually would not survive these conditions much longer than 5 minutes, the animals were sacrificed after 5 minutes.

It must be admitted that, even under such comparable conditions, the physiological effects of a lack of oxygen may vary in the individual animals. In fact, we observed gasping in a few control animals (not in any of the injected animals), even before the expiration of the 5 minutes. These non-injected animals were sacrificed just before the expiration of the 5 minute period when it was evident that they would not survive the full period. If any errors were introduced by such a shortening of the period of anoxia, it would be in the direction of diminishing the effect of the anoxia in the non-injected animals, and hence would make the effect of the cytochrome *c* less striking than otherwise. Actually there was no significant difference in the findings in these few animals and, even if these findings were discarded, the general results of our experiments would remain the same. There can be no doubt about the correctness of the inference that the great majority of the animals had reached an advanced stage of anoxia when they were sacrificed. Our observation that some control animals began gasping even before the 5 minute period had expired can only be considered as a corroboration of this conclusion. The fact that in only one of the uninjected animals under anoxia were adenyl pyrophosphate figures observed approaching the average values under normal conditions proves that the experimental conditions applied in this investigation were sufficiently severe to justify the assumption that consistent differences observed between the injected and control animals were caused by the cytochrome injection rather than by any possible accidental variations in the degrees of anoxia.

Since requirements of the chemical analysis permitted the use of only a small number of animals in each group, a statistical treatment of the results obtained in the single groups would not be possible. For this reason, the



division into groups has been omitted in the presentation of our results in Table I.

There was no significant difference in activity in the control and injected rats during the period of anoxia.

TABLE I

*Effect of Anoxia Alone and after Intravenous Injection of Cytochrome c on Easily Hydrolyzable Phosphorus Fraction in Heart and Kidney of Rats*

The results are expressed in mg. of easily hydrolyzable P in 100 gm. of fresh tissue.

Experiment No.	Heart			Kidney		
	Normal animals	Animals in 3 per cent O <sub>2</sub>	Animals in 3 per cent O <sub>2</sub> after injection of 5 mg. cytochrome c	Normal animals	Animals in 3 per cent O <sub>2</sub>	Animals in 3 per cent O <sub>2</sub> after injection of 5 mg. cytochrome c
1	19.9	6.1	12.9	9.7	4.0	8.2
2	14.5	3.4	14.7	8.2	5.6	7.5
3	16.8	8.4	7.4	12.3	3.9	7.5
4	6.4	7.3	13.1	7.6	3.8	2.2
5	19.3	6.7	10.4	6.7	3.8	6.0
6	6.4	3.7	10.5	6.1	4.4	6.1
7	8.6	8.4	10.6	6.1	2.5	3.2
8	14.9	11.0	12.2	7.5	6.5	7.0
9	16.9	5.4	15.9	6.5	3.8	5.4
10	13.1	9.2	13.3	7.2	3.1	6.3
11	10.5	9.2	13.0	7.5	5.3	8.0
12	18.9	8.8		6.8	4.7	6.6
13	18.7	8.8		9.9	3.0	
14	14.7	5.1		8.4	3.5	
15	14.8	5.1		9.4	3.7	
16	15.3			9.3	3.4	
17	15.3			5.5	3.5	
18	10.9			7.8	3.7	
19				3.6	3.6	
20				7.7	1.8	
21				6.8		
Average.....	14.2	7.1	12.1	7.6	3.8	6.1
Probable deviation from average.....	0.73	0.38	0.44	0.195	0.155	0.36

### Results

Table I shows (a) the contents of easily hydrolyzable phosphorus fraction in hearts and kidneys of normal rats, (b) the content after 5 minutes of anoxia, and (c) the content after 5 minutes of anoxia when 5 mg. of cytochrome c had been previously injected intravenously.

It is evident in the first place that a sharp reduction of the easily hydrolyzable phosphorus fraction results from anoxia, and secondly that this reduction can be largely prevented by the previous administration of cytochrome *c*. Incidentally, we found that the previous injection of adenylyl pyrophosphate had no such effect. The average value for the normal heart is 14.2 mg. per cent and for the normal kidney 7.6 mg. per cent. The average values in those organs after anoxia are 7.1 and 3.8 mg. per cent respectively. The average value in the organs of cytochrome *c*-treated animals after anoxia is 12.1 mg. per cent in the heart and 6.1 mg. per cent in the kidney.

It can be seen from Table I that the differences in the normal and anoxic organs on the one hand and the anoxic and cytochrome *c*-treated organs on the other hand are sufficiently striking to be well outside the range of accidental deviations. This is confirmed by statistical analysis, the results of which are recorded in Table I.

#### DISCUSSION

The easily hydrolyzable phosphorus fraction is known to consist of substances which participate in cell metabolism with a considerable rate of turnover. The organ content of this fraction must therefore be considered to be the result of a dynamic equilibrium. Our findings tend to indicate that this equilibrium is easily influenced by anoxia.

It has been shown in enzyme experiments *in vitro* that the resynthesis of adenylyl pyrophosphate is coupled with oxidative processes (7). The results of our experiments suggest that this situation obtains in the intact animal as well. The exact mechanism by which cytochrome *c* prevents the depletion of the reserves of the easily hydrolyzable phosphorus fraction resulting from anoxia is not known. One possible explanation is that the added cytochrome *c* is capable of maintaining the rate of oxidative processes at a sufficiently high level so as to achieve an almost normal degree of resynthesis of adenylyl pyrophosphate, despite the anoxia.

The possibility of thus employing cytochrome *c* parenterally to offset some of the harmful effects of the chemical changes resulting from anoxia is obvious. We have in fact been able to demonstrate that a number of clinical effects of anoxia can be thus prevented (2).

#### SUMMARY

1. The content of the easily hydrolyzable phosphorus fraction of the hearts and kidneys of normal rats was determined under conditions of normal and low oxygen tension. Under low oxygen tension, there was a sharp decrease in the content of this fraction.

2. This decrease could be largely prevented by the previous intravenous injection of cytochrome *c*.

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# A NOTE ON THE ELECTROPHORETIC PROPERTIES OF DENATURED SERUM ALBUMIN

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When horse or beef serum albumin is denatured by concentrated solutions of urea or guanidine hydrochloride, and the denaturing agent is subsequently removed by dialysis, two products of widely different physico-chemical properties are obtained (1, 2). One of these, designated as "apparently reversibly denatured" or "regenerated" serum albumin, resembles the native protein in solubility, in molecular size and shape, and in electrophoretic behavior (2, 3), but differs in crystallizing ability and susceptibility to tryptic hydrolysis (2, 4). The other fraction, believed to represent truly "irreversibly denatured" albumin, is highly insoluble at the isoelectric point and was found to be polydisperse when dissolved in neutral solution (pH 7.1). The high viscosity exhibited in acidic solutions (pH 4), as well as retarded and anomalous diffusion, precluded a determination of its molecular kinetic properties (1). Analogous findings have been observed with the products obtained after denaturation by concentrated urea solutions of horse serum pseudoglobulin GI (4-6).

In view of the molecular heterogeneity of aqueous solutions of irreversibly denatured serum albumin, obtained after denaturation by urea or guanidine hydrochloride as well as after denaturation by heat (7), it was deemed of interest to investigate the electrophoretic properties of a representative fraction. Crystalline, carbohydrate-free beef albumin<sup>1</sup> was chosen because of its standardized properties and the greater amount of irreversibly denatured fraction it yields (2) as compared to crystalline horse serum albumin.

A 2 per cent solution of beef serum albumin was denatured by 6 M guanidine hydrochloride.<sup>2</sup> After standing for 48 hours at room temperature, preliminary separation of the soluble regenerated and the poorly soluble irreversibly denatured fractions was carried out by removing the denaturant

<sup>1</sup> Obtained through the courtesy of Dr. E. J. Cohn, Department of Physical Chemistry, Harvard Medical School, and Dr. H. B. Vickery. The preparation was made under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

<sup>2</sup> A solution of 9 per cent beef albumin in 8 M guanidine hydrochloride at pH 7.4, on standing overnight at room temperature, sets to a stiff gel distributed throughout with threads of denatured protein.

by dialysis and heating the suspension at pH 5.25 at 41° for 30 minutes. After centrifugation, electrophoretic analysis of the clear supernatant solution was made in a veronal-NaCl buffer, pH 7.6,  $\mu = 0.1$  (0.03 M sodium veronal, 0.07 M NaCl), with the apparatus and technique previously described (6).

It was found that the initially homogeneous albumin (Fig. 1, Experiment 1) was now about equally distributed between two components (Fig. 1, Experiment 2), one of these having nearly the same mobility as the native protein, whereas the mobility of the other one was less.

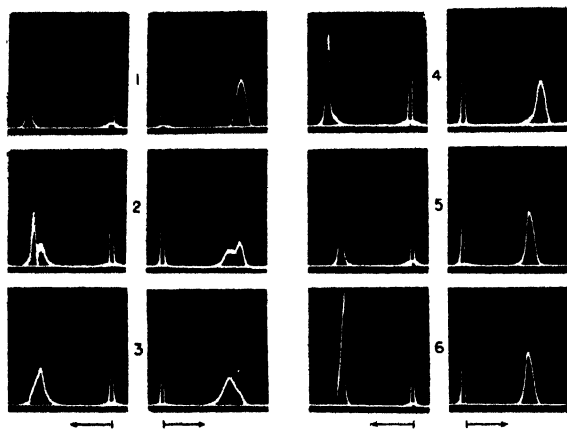


FIG. 1. Electrophoretic patterns of native, irreversibly denatured, and regenerated beef serum albumin. For the conditions of Experiments 1 to 6 and identification of components, see the text and Table I. For each experiment, the right hand diagram represents the descending pattern, the left-hand diagram the ascending pattern. The arrows indicate the direction of migration

Fractional precipitation of the proteins of this solution with ammonium sulfate, at pH 4.8, yielded two products, one precipitating upon addition of the solid salt up to a concentration of 2.0 M, the other one precipitating between 2.0 and 2.35 M. Electrophoretic analysis revealed the less soluble fraction to consist predominantly of the slower component (Fig. 1, Experiment 3), whereas the more soluble fraction is composed almost wholly of a component having the mobility of the native albumin (Fig. 1, Experiment 4). The mobility values are given in Table I.

In order to determine the electrophoretic properties of the irreversibly denatured fraction, the precipitate obtained in the preliminary separation upon heating of the salt-free solutions at 41° was washed with distilled water and dissolved by the addition of 0.5 N NaOH to pH 7.8. After reprecipitation by readjustment to pH 5.25, with HCl, the precipitate was again dis-

solved at pH 7.8, yielding a highly viscous, opalescent solution. Electrophoresis revealed a single, sharp boundary of essentially the same mobility as that of the slow moving, less soluble fraction described above (Fig. 1, Experiment 5, Table I). Photographs of the boundaries, taken after more than 15 hours standing with the current off, indicated an extremely slow rate of diffusion (Fig. 1, Experiment 6).

The component with the higher mobility ( $6.4 \times 10^{-5}$  sq. cm. per second per volt) is believed to represent regenerated albumin. This deduction is made in analogy with the behavior of *whole* beef albumin which, on regeneration after denaturation by guanidine hydrochloride (but not by urea), exhibits the same mobility as the native protein, though differing somewhat

TABLE I  
*Electrophoretic Analysis of Native, Irreversibly Denatured, and Regenerated Beef Serum Albumin\**

Experiment No.	Protein	Time  sec.	Mobility,† $10^{-5}$ sq. cm. volt <sup>-1</sup> sec. <sup>-1</sup>	
			Denatured	Native or regenerated
1	Native	14,580		-6.42
2	Supernatant, unfractionated	14,580	-5.49	-6.33
3	“ 1st fraction	14,400	-5.54	
4	“ 2nd “	14,400		-6.40
5	Isoelectric ppt.	14,400	-5.37	

\* All experiments were performed at 1° in a veronal-NaCl buffer, pH 7.6,  $\mu = 0.1$  (0.03 M sodium veronal, 0.07 M NaCl), at a constant field strength varying from experiment to experiment between 5.55 and 5.64 volt per cm.

† Data for descending boundary only.

in molecular kinetic properties and ease of tryptic hydrolysis (2). Since the slow component obtained by fractional precipitation from mixtures has the same mobility as the protein solution prepared from the isoelectric precipitate, it may be identified as irreversibly denatured albumin.

It is of interest that the presence of two components, one of lower mobility (denatured), the other of mobility exceeding that of the native protein (regenerated?), has also been observed upon heating horse serum albumin (7), though the quantitative distribution was found to depend greatly on ionic strength. Moreover, a change occurs in the electrophoretic pattern of both horse serum albumin (8) and hen's egg albumin on aging (9). In originally electrically homogeneous horse serum albumin, aging produces a second component of lowered electric mobility and diffusion constant, this change being accelerated by heating (8). Likewise, the ratio of the two

components of freshly prepared hen's egg albumin is altered with age, conversion to a soluble, crystallizable, slow moving component being complete on prolonged standing (9).

The present study shows that, although denaturation of serum albumin (as of many other proteins (10)) may lead to an over-all increase in molecular kinetic heterogeneity, it is possible to isolate the irreversibly denatured product in an electrophoretically homogeneous form. It is recognized that denaturation may produce small changes in amphoteric properties of proteins, as revealed by an alkaline shift of the isoelectric point, pH-mobility curves, and titration data (10). The available evidence suggests that these changes result from the liberation of paired acidic and basic groups upon the rupture of structure-determining hydrogen bonds and that the resultant change in molecular configuration allows for a spatial redistribution of polar groups with exposure of non-polar residues. The recognized tendency of denatured proteins towards aggregation is in accord with these views.

This work has been carried out with the support of the Rockefeller Foundation, the Lederle Laboratories, Inc., and the Duke University Research Council.

#### SUMMARY

Electrophoretic analysis has been made on the products obtained after removal of the denaturing agent from solutions of crystalline beef albumin in 6 M guanidine hydrochloride. It is shown that isolation of the irreversibly denatured protein, either by ammonium sulfate fractionation or by isoelectric precipitation, yields electrophoretically homogeneous material, characterized by retarded electric mobility and diffusion rate. The relation of the observed components to regenerated and irreversibly denatured protein is discussed.

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## THE VERATRINE ALKALOIDS

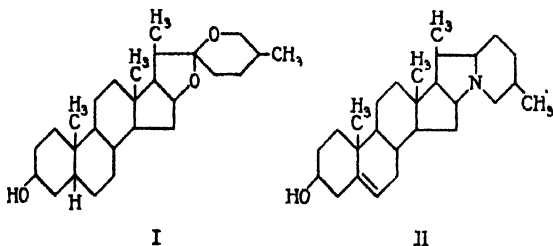
### XXIV. THE OCTAHYDROPYRROCOLINE RING SYSTEM OF THE TERTIARY BASES. CONVERSION OF SARSASAPOGENIN TO A SOLANIDINE DERIVATIVE

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(Received for publication, June 12, 1945)

The *Solanum* and *Veratrum* alkaloids have been shown to be steroid or, possibly in the case of certain individual veratrine bases, modified steroid derivatives, in which the heterocyclic portion of the molecule has been assumed to involve the usual iso-octyl side chain (1). These alkaloids fall into the two groups of secondary and tertiary bases, both of which give the characteristic 2-ethyl-5-methylpyridine on dehydrogenation. Since the postulated structures for these substances have been inferred entirely from transformation and degradation experiments, from the formation of digitonides, and from physical measurements (2), the present investigation was undertaken in an attempt to obtain direct evidence by partial synthetic procedures.

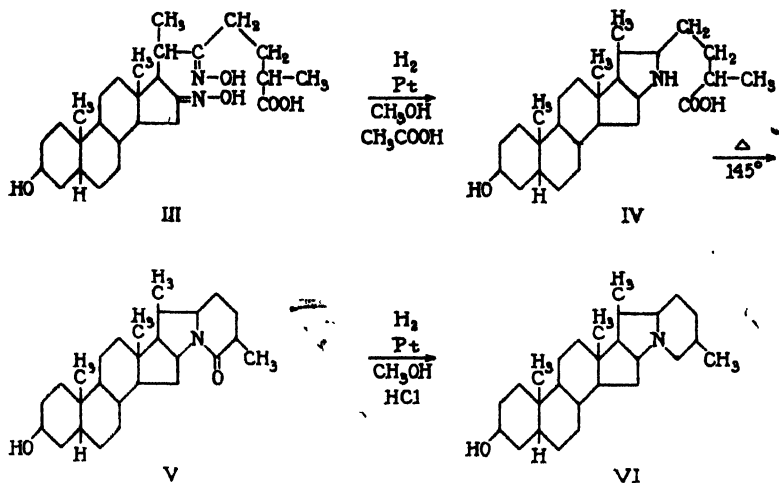


A promising starting material for this purpose was thought to be certain naturally occurring steroid sapogenins, such as sarsasapogenin. The formula (I) for the latter substance suggests a possible manner in which the steroid alkaloids could be built up, for if the oxygen atoms of the distinctive spiroketal side chain were replaced by a nitrogen atom, the suggested structure (II) for solanidine, for example, would result. In the experiments herein reported, it has been possible to convert sarsasapogenin (I) directly to the stereochemically corresponding dihydrosolanidine. This substance has recently been described under the name allosolanidanol-(3 $\beta$ ) by Prelog and Szpilfogel, who have prepared the four stereoisomeric dihydrosolanidines with the *cis* and *trans* configurations of Rings A and B of both the 3 $\alpha$ (OH) and 3 $\beta$ (OH) series. This definitely establishes



the structure (II) for solanidine, and since the veratrine alkaloids give the same basic degradation product, 2-ethyl-5-methylpyridine, and, in general, are structurally analogous to solanidine, the formulation of the heterocyclic portion of the molecule in the tertiary bases of the veratrine series as an octahydropyrrocoline derivative may be considered quite certain.

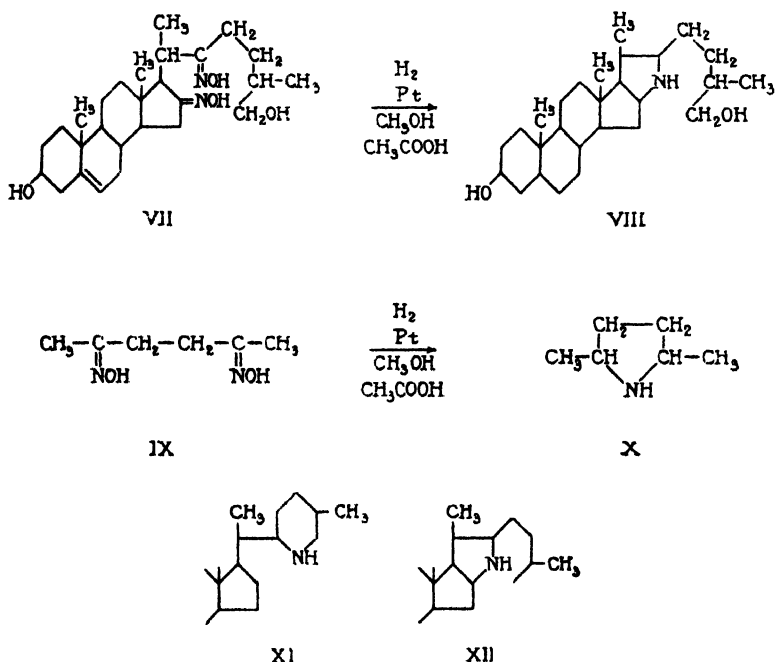
The starting point for the transformation of the sapogenin to the alkaloid series was the diketo acid, sarsasapogenoic acid, obtained by the oxidation of sarsasapogenin acetate with chromic acid in acetic acid solution (3). Sarsasapogenoic acid dioxime (III) was hydrogenated with platinum oxide in a methanol-acetic acid solution. From the ampholyte fraction of the complex mixture of reduction products a secondary *amino acid* of structure (IV) was isolated. This substance gave an *N*-nitroso



derivative. The amino acid lactamized readily when heated to give the *neutral lactam* (V). The lactam grouping proved to be stable to reduction with sodium and butanol, with phosphorus and hydriodic acid, and with copper chromite and hydrogen at elevated temperature and pressure. However, it was readily reduced to the *tertiary amine* with an active platinum oxide catalyst in dilute hydrochloric acid in methanol (4). The *lactam* (V) when submitted to hydrogenation under the latter conditions gave a tertiary base which was shown to be identical with allosolanidanol-(3β) (VI). The latter was prepared from solanidine, according to Prelog and Szpilfogel, by oxidation with aluminum tertiary butoxide, followed by reduction of the unsaturated ketone with platinized Raney's nickel, and subsequent separation of the epimers with digitonin (5). Identity was confirmed by comparison of the corresponding acetates.

In another approach to the problem of the conversion of a sapogenin to

the nitrogenous derivatives, begun simultaneously with the above work, the hydrogenation of cryptogenin dioxime was studied. Bethogenin (6) was prepared from the roots of *Trillium erectum* and converted to cryptogenin dioxime (VII) with hydroxylamine (7). Hydrogenation of cryptogenin dioxime in methanol-acetic acid solution gave the *secondary amine* of structure (VIII). The base was characterized as the *nitroso derivative*. Repeated attempts, however, to eliminate water between the secondary nitrogen and the terminal primary alcoholic group were unsuccessful.



Preliminary efforts to prepare the mono- or dihalogen derivatives of the base likewise proved unpromising.

Acetonylacetone dioxime (IX) was used as a model compound for study of the behavior of 1,4-dioximes under various reductive procedures. When submitted to hydrogenation under the conditions successfully employed for the reduction of sarsasapogenoic acid and cryptogenin dioximes, acetonylacetone was converted to 2,5-dimethylpyrrolidine (X) in good yield. The product was isolated and characterized as the hydrochloride.

Finally, the exact structure of the heterocyclic side chain in these secondary bases of the veratrine series remains to be established. Since jervine has also yielded 2-ethyl-5-methylpyridine on dehydrogenation (8), the arrangement suggested in formula (XI), rather than the alternative

formula (XII), would appear to be the probable skeletal structure for the heterocyclic portion of these alkaloids.

#### EXPERIMENTAL

*Hydrogenation of Sarsasapogenoic Acid Dioxime*—A suspension of 1.0 gm. (0.0084 mole) of sarsasapogenoic acid dioxime (3) in a mixture of 250 ml. of methanol and 15 ml. of acetic acid was shaken with hydrogen and 0.50 gm. of platinum oxide at ordinary temperature and pressure. After absorption of 310 ml. of hydrogen during 40 hours (theory 330 ml.) the hydrogenation was interrupted and the filtrate from the catalyst was concentrated to a syrup *in vacuo*. A chloroform solution of the residue was extracted repeatedly with water and the combined washings were evaporated to dryness *in vacuo*. The solid residue was extracted with dilute ammonium hydroxide and a small amount of insoluble material was removed by filtration. The ammoniacal filtrate was evaporated to dryness *in vacuo*, and the amino acid thus liberated was recrystallized from dilute ethanol. The yield was 90 mg. (10 per cent). The amino acid melted at 143° with loss of water and the melt at once crystallized to form the lactam.

$$[\alpha]_D^{25} = +25.0^\circ \text{ (} c = 2.0 \text{ in ethanol)}$$

$C_{27}H_{46}O_2N$ . Calculated, C 75.13, H 10.51, N 3.25; found, C 74.90, H 10.72, N 3.47

The *nitroso derivative* was prepared by addition of aqueous sodium nitrite to a solution of the amine in dilute acetic acid. It was extracted with chloroform and recrystallized from dilute ethanol. M.p., 160–162°.

$C_{27}H_{44}O_4N_2$ . Calculated, C 70.40, H 9.63, N 6.08; found, C 70.25, H 9.61, N 6.00

The *Lactam*, ( $C_{27}H_{43}O_2N$ ), was prepared by heating the dry amino acid. The ampholyte melted at 143° and the melt crystallized at once to form the lactam, which was recrystallized from dilute ethanol. The yield was practically quantitative. M.p., 200–202°.

$$[\alpha]_D^{25} = +17.0^\circ \text{ (} c = 1.4 \text{ in ethanol)}$$

$C_{27}H_{43}O_2N$ . Calculated, C 78.40, H 10.53, N 3.39; found, C 78.63, H 10.46, N 3.50

*Reduction of Lactam (V)*—A solution of 175 mg. (0.00042 mole) of the lactam (V) in a mixture of 15 ml. of methanol and 5 ml. of 10 per cent hydrochloric acid was hydrogenated at ordinary temperature and pressure in the presence of 175 mg. of platinum oxide. After 65 ml. of hydrogen had been absorbed during a period of 15 hours (theory 66 ml.), the catalyst was removed and the filtrate was concentrated to dryness *in vacuo*. The residue was treated with dilute sodium hydroxide solution and the mixture was extracted with chloroform. The washed and dried extract was concentrated *in vacuo*. The crystalline residue was treated

with dilute acetic acid, a small amount of insoluble material was removed by filtration, and the amine was precipitated by addition of dilute sodium hydroxide solution to the filtrate. After recrystallization from ethanol, the yield was 70 mg. (41 per cent). M.p., 216–218°. The mixed melting point with allosolanidanol-(3 $\beta$ ) (m.p., 216–218° (5)) was 216–218°. The mixed melting point with solanidanol-(3 $\beta$ ) (m.p., 219–220°) was 188–192°.

$$[\alpha]_D^{25} = +27.3^\circ (c = 1.2 \text{ in chloroform})$$

Prelog and Szpilfogel reported  $[\alpha]_D^{17} = +27.9^\circ (c = 0.81) (5)$ .

$C_{27}H_{46}ON$ . Calculated, C 81.14, H 11.35, N 3.51; found, C 80.95, H 11.50, N 3.56

*Acetylation of the Amine*—A solution of 50 mg. of the above base in a mixture of 1 ml. of pyridine and 1 ml. of acetic anhydride was allowed to stand at room temperature for 15 hours. The solvents were then removed *in vacuo*. The residue was treated with dilute ammonium hydroxide solution and the solid was collected with water, and dried. It was recrystallized from ethanol. M.p., 144–146°. The mixed melting point with allosolanidanol-(3 $\beta$ ) acetate (m.p., 144–146° (5)) was 144–146°.

$$[\alpha]_D^{25} = +28^\circ (c = 0.57 \text{ in chloroform})$$

Prelog and Szpilfogel reported  $[\alpha]_D^{19} = +31.4^\circ (c = 0.605) (5)$ .

$C_{25}H_{47}O_2N$ . Calculated, C 78.86, H 10.73, N 3.17; found, C 78.94, H 10.87, N 3.29

*Hydrogenation of Cryptogenin Dioxime*—A solution of 200 mg. (0.00043 mole) of cryptogenin dioxime (6) in a mixture of 50 ml. of methanol and 3 ml. of acetic acid was shaken with hydrogen and 100 mg. of platinum oxide at ordinary temperature and pressure. After 70 ml. of hydrogen had been absorbed during 15 hours (theory 75 ml.), the filtrate from the catalyst was concentrated *in vacuo*. The residue was treated with ether and the ether solution was extracted with several successive portions of water. The amine crystallized when dilute ammonium hydroxide was added to the combined aqueous extracts. It was recrystallized from dilute ethanol. The yield was 60 mg. (33 per cent). M.p., 177–182°.

$C_{27}H_{47}O_2N$ . Calculated, C 77.64, H 11.34, N 3.35; found, C 77.70, H 11.50, N 3.53

*Nitroso Derivative*—The nitroso derivative separated in crystalline form when a sodium nitrite solution was added to a solution of the amine in dilute acetic acid. The yield was essentially quantitative. M.p., 213–215°.

$C_{27}H_{46}O_3N_2$ . Calculated, C 72.60, H 10.38, N 6.27; found, C 72.70, H 10.10, N 6.45

*Hydrogenation of Acetonylacetone Dioxime*—A solution of 1.44 gm. (0.01 mole) of acetonylacetone dioxime (9) in a mixture of 250 ml. of methanol

and 15 ml. of glacial acetic acid was shaken with hydrogen and 200 mg. of platinum oxide at ordinary temperature and pressure. Absorption ceased after 940 ml. had been taken up during 20 hours (theory 1030 ml.). The filtered solution was concentrated to a few ml. *in vacuo*. Sodium hydroxide was added in excess and the mixture was extracted with a large volume of ether. The dried extract was concentrated to a small volume and dry hydrogen chloride was passed in to precipitate the hydrochloride. The crystalline salt was recrystallized from absolute ethanol and ether. The yield was 750 mg. (55 per cent). M.p., 205–208° (10).

$C_{24}H_{31}NCl$ . Calculated, C 53.13, H 10.40, N 10.33; found, C 53.13, H 10.15, N 10.20

All melting points are micro melting points.

All analytical data recorded were obtained by Mr. D. Rigakos.

#### SUMMARY

Sarsasapogenin has been converted to the stereochemically corresponding dihydrosolanidine, allosolanidanol-(3 $\beta$ ). The heterocyclic portion of the molecule in the tertiary bases of the *Solanum* and *Veratrum* alkaloid series is an octahydropyrrocoline derivative formed by condensations which involve carbon atoms 22 and 26 of the isoetyl steroid side chain and carbon atom 16 with nitrogen.

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# THE EFFECT OF CAFFEINE ON UREA FORMATION FROM AMMONIUM SALTS BY LIVER SLICES IN VITRO

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Very little work has been done on the specific metabolic effects of caffeine. It is known that caffeine increases the basal metabolic rate and that high concentrations cause contracture in skeletal muscle. The earlier workers investigated the nitrogen excretion in the urine after varying and, in most cases, unspecified amounts of caffeine had been administered. Their results were contradictory. Lehmann (1) found less nitrogen in human urine when caffeine was given and Rabuteau (2) found a significant decrease in urea excretion. On the other hand, Hoppe (3) and Voit (4) could demonstrate little effect on the total nitrogen and urea excretion in dogs, and Roux (5) was unable to confirm Rabuteau's results in man. Farr and Welker (6) gave small doses of caffeine to two men. There was little effect on urea excretion but in one individual an increased ammonia excretion occurred. In the past 30 years this aspect of the problem has apparently been neglected. In the following report, the effect of caffeine on the metabolism of liver and kidney slices *in vitro* is described. In both tissues caffeine interferes with the utilization of added ammonium salts and in liver it inhibits the formation of urea.

## EXPERIMENTAL

For most of the experiments the liver and kidney of approximately 250 gm. rats were used. 300 mg. (wet weight) of slices were suspended in 4.0 cc. of Krebs' bicarbonate solution in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide and were shaken for 3 to 4 hours at 37°. At the end of the experiment 1.0 cc. of 20 per cent trichloroacetic acid was added to each vessel and the precipitated protein centrifuged. Aliquots of the supernatant fluid were taken for the estimation of urea, ammonia, and amino nitrogen. Urea was estimated by the method of Ormsby (7) and of other substances liable to be present in tissue filtrates only allantoin in high concentration gives a color similar to that of urea with the biacetyl reagent. Experiments with allantoin production by liver slices from hypoxanthine and uric acid showed that caffeine was without effect on this reaction. The red color produced when citrulline reacts with the biacetyl reagent

was not observed. The ammonia was estimated by vacuum distillation from 5 per cent NaOH at 60° for 5 minutes, and subsequent nesslerization. The color was read in an Evelyn photocolormeter with a No. 420 filter. In this procedure a small amount of amide nitrogen is estimated, but this was an interfering factor only when glutamine was used. Volatile amines produce either little color or a milky cloudiness with Nessler's reagent, and cloudiness was not observed in our experiments. By the Van Slyke method (8) for amino nitrogen, under our conditions, 40 per cent of the ammonia present and 6 per cent of the urea are also estimated. Calculations for this were made. Extra nitrogen is evolved in the presence of acetoacetic acid, but this compound is not involved in the caffeine effect, for boiling the filtrate with acid for 30 minutes did not change the effect of caffeine on the nitrogen values.

The first experiments consisted of amino nitrogen determinations on the trichloroacetic acid filtrates of liver slices. When the slices were incubated with caffeine, an average of 30 per cent less amino nitrogen was present than in the controls. Thus in a typical experiment 0.248 mg. of  $\text{NH}_2\text{-N}$  was found in the controls, and only 0.175 mg. in the presence of  $5.7 \times 10^{-3}$  M caffeine. This effect was remarkably consistent, the decrease varying from 22 to 39 per cent. Higher concentrations of caffeine did not increase the effect. On the other hand, caffeine produced a 10 per cent decrease when incubated with the same weight of kidney slices under the same conditions and had a negligible effect on the amino nitrogen in filtrates from heart slices. When liver slices were incubated anaerobically, caffeine had no effect. These results indicated that caffeine has some specific action on the nitrogen metabolism of liver and that it was not merely affecting the permeability of the tissue slices. Possibly, caffeine was accelerating the deamination of amino acids; so a number of amino acids were incubated with liver slices. These included glycine, alanine, serine, methionine, valine, leucine, isoleucine, arginine, and phenylalanine, and both the natural and non-natural isomers were used. In no instance did caffeine have any effect on the rate of deamination.

In the next experiments, ammonium sulfate was incubated with liver slices. Since ammonia is estimated by the Van Slyke procedure, the decrease in apparent amino nitrogen in the presence of caffeine might have been due to an increased utilization of or a decreased formation of amino nitrogen from ammonia. The results given in Table I show that caffeine apparently interferes with the utilization of ammonia, and suggest that it might be inhibiting urea production from ammonia. Urea determinations were made and the results of a typical experiment are given in Table I. Caffeine definitely inhibits urea formation from ammonia, the inhibition varying in different experiments from 25 to 52 per cent. The concentration

of caffeine for maximum inhibition varied from  $3.0$  to  $6.0 \times 10^{-3}$  M, and larger concentrations were without greater effect. 4.0 mg. of ammonium

TABLE I

*Effect of  $5.7 \times 10^{-3}$  M Caffeine on Urea Production and Ammonia and Amino Nitrogen Metabolism in Liver Slices, and on Latter Two in Kidney Slices*

300 mg. (wet weight) of tissue were shaken 3 hours at  $37^\circ$  in 4.0 cc. of Krebs' bicarbonate solution in 95 per cent  $O_2$ -5 per cent  $CO_2$ .

Experiment No.	Rat tissue	Compounds added	Urea formed	NH <sub>2</sub> -N recovered	Apparent NH <sub>2</sub> -N recovered*
			mg.	mg	mg.
1	Liver	None	0.22	0.194	0.248
		2.0 mg. dl-ornithine	0.28	0.094	0.505
		4.0 " (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.98	0.325	0.405
		4.0 " " + 2.0 mg. dl-ornithine	1.39	0.172	0.615
		4.0 " caffeine	0.20	0.161	0.175
		4.0 " " + 2.0 mg. dl-ornithine	0.21	0.094	0.485
		4.0 " " + 4.0 " (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.67	0.490	0.425
		4.0 " " + 4.0 " " + 2.0 mg. dl-ornithine	1.27	0.158	0.590
2	"	None	0.30	0.130	
		2.0 mg. glutamine	0.38		
		2.0 " dl-ornithine	0.34	0.120	
		4.0 " (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.26	0.302	
		4.0 " " + 2.0 mg. glutamine	1.40		
		4.0 " " + 2.0 " dl-ornithine	1.57	0.156	
		4.0 " caffeine	0.29	0.118	
		4.0 " " + 2.0 mg. glutamine	0.31		
		4.0 " " + 2.0 " dl-ornithine	0.29	0.118	
		4.0 " " + 4.0 " (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.46	0.630	
		4.0 " " + 4.0 " " + 2.0 mg. glutamine	1.21		
		4.0 mg. caffeine + 4.0 mg. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + 2.0 mg. dl-ornithine	1.18	0.491	
3	Kidney	None		0.138	0.212
		4.0 mg. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		0.705	0.505
		4.0 " caffeine		0.107	0.195
		4.0 " " + 4.0 mg. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		0.954	0.510

\* These figures include 40 per cent of the remaining NH<sub>2</sub>-N, 6 per cent of the urea formed, the tissue blank as well as the NH<sub>2</sub>-N. For calculations of actual NH<sub>2</sub>-N see the text.

sulfate containing 0.85 mg. of nitrogen were found the most suitable concentration to use in these experiments. Ammonia determinations, also given in Table I, show that with the inhibition of urea production there is



an accumulation of ammonia. Table II shows the effect of different concentrations of caffeine.

In order to determine how caffeine interferes with urea formation the following experiments were done. 1.0 mg. of arginine was added to liver slices and 0.36 mg. of urea was formed from it. In the presence of caffeine 0.32 mg. was formed. Therefore caffeine does not inhibit arginase. When 2.0 mg. of *dl*-ornithine were added to liver slices, there was some increase of urea production and ornithine with ammonia always causes a urea formation greater than the sum of the urea from ornithine and ammonia separately. As shown in Table I the presence of ornithine almost completely overcomes the inhibition of urea formation from ammonia by caffeine. If glutamine is added to liver slices, a slight increase of urea production is obtained, but, unlike ornithine, glutamine does not increase

TABLE II

*Effect of Various Concentrations of Caffeine on Urea Formation and Ammonia Metabolism by 300 Mg. (Wet Weight) of Rat Liver Slices Shaken for 3.5 Hours at 37° in 95 Per Cent Oxygen and 5 Per Cent Carbon Dioxide*

The control values have been subtracted.

	Urea formed	NH <sub>3</sub> -N recovered
	mg.	mg.
4.0 mg. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	0.82	0.26
4.0 " " + 0.7 × 10 <sup>-3</sup> M caffeine.....	0.71	0.32
4.0 " " + 2.1 × 10 <sup>-3</sup> " ".....	0.61	0.34
4.0 " " + 5.7 × 10 <sup>-3</sup> " ".....	0.52	0.43

the urea formation from added ammonia. However, glutamine does restore urea formation from ammonia when this process has been inhibited by caffeine (Table I). Glutamic acid is without effect.

Thus caffeine interferes in some way with the formation of urea but if sufficient amounts of ornithine or glutamine are present the inhibition can be overcome. It is not possible to state exactly how this inhibition is brought about, and it may be that caffeine interferes in general with the utilization of ammonia. Thus if less ammonia is converted to amino nitrogen in the presence of caffeine the lower Van Slyke values could be accounted for. The following calculation indicates that actually less amino nitrogen is formed from added ammonia in the presence of caffeine. If, from the values obtained by the Van Slyke method, which include the amino nitrogen and 40 per cent of the ammonia, 40 per cent of the ammonia (estimated by distillation) is subtracted, then the amino nitrogen can be estimated. Filtrates from control liver slices by this calculation contain 0.140 mg. of

NH<sub>2</sub>-N; in the presence of caffeine, 0.121 mg. When ammonia is added, the corresponding values are 0.275 and 0.229 mg. Filtrates from control kidney slices contain 0.167 mg.; in the presence of caffeine, 0.152 mg.; and after the addition of ammonia, 0.223 and 0.160 mg. It would therefore appear that in the presence of caffeine less ammonia is converted to compounds that will react with nitrous acid.

Theophylline and theobromine act similarly to caffeine, although because of the relative insolubility of theobromine its effects are not marked. The inhibition of urea formation by caffeine can also be demonstrated in rabbit, guinea pig, and dog liver.

#### DISCUSSION

Since the caffeine inhibition of urea formation from ammonia is so definite *in vitro*, it will be of interest to determine whether it also occurs *in vivo*. That some of the earlier workers found a low urea excretion after caffeine and others did not indicates that certain conditions may be necessary before the effect is demonstrable in the whole animal. The fact that caffeine can interfere with the nitrogen metabolism of the liver and the kidney may eventually explain its effects on the basal metabolic rate and on the production of prothrombin, as recently shown by Field *et al.* (9).

#### SUMMARY

1. Caffeine inhibits the formation of urea from ammonia in liver. The addition of ornithine or glutamine overcomes the inhibition. Caffeine has no effect on arginase.

2. In both liver and kidney caffeine inhibits the disappearance of added ammonia. In the liver this is explained by the decrease in urea formation and a decrease in amino nitrogen formation, in the kidney by a decrease in amino nitrogen formation.

3. Theophylline and theobromine have similar effects.

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# ISOLATION OF ANDROSTERONE, ETIOCHOLAN-3( $\alpha$ )-OL-17-ONE, AND $\Delta^5$ -ANDROSTENE-3( $\beta$ ),17( $\alpha$ )-DIOL FROM THE URINE AFTER ADMINISTRATION OF DEHYDROISOANDROSTERONE TO A MAN

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Dehydroisoandrosterone is normally a minor fraction of the urinary ketosteroids. Callow and Callow (1) isolated from the urine of normal men and women about 0.2 mg. per liter, as compared with 1.3 to 1.6 mg. of androsterone and 1.3 to 1.4 mg. of etiocholan-3( $\alpha$ )-ol-17-one. The urine of most patients who have tumors of the adrenal cortex, however, contains more dehydroisoandrosterone (2-4) than any other steroid. The total amount excreted daily may be several hundred mg. Our interest in the metabolism of this substance was aroused by speculation as to whether dehydroisoandrosterone is one of the end-products of steroid metabolism or whether it is capable of transformation to some of the other steroids that are excreted in the urine. Since the metabolism of dehydroisoandrosterone has received very little attention, this study was undertaken to determine what urinary steroids might be formed when dehydroisoandrosterone was administered to a human subject.

In order to avoid the uncertainties which would arise from the steroids which are normally present in the urine, a man was selected for this study whose excretion of steroids was very small, 1.0 to 2.1 mg. in 24 hours. This man, 30 years of age, had had a pituitary tumor removed at the age of 25 years. He presented the classical clinical picture of severe anterior pituitary insufficiency with marked secondary gonadal, thyroid, and adrenal failure, although symptoms of secondary adrenal cortical insufficiency were less evident. In view of what is known about the anatomic status of the adrenal cortex in other cases of this type there is little doubt that the adrenal cortical function was impaired. The low level of the urinary 17-ketosteroids in itself is evidence in favor of this view.

The dehydroisoandrosterone was given in the form of the acetate, which is more soluble in oil than the alcohol. The acetate was dissolved, 25 mg. in 1 ml., in peanut oil which contained 2 per cent of benzyl alcohol to increase the solubility. It was injected intramuscularly in two doses daily of 25 mg. each for 7 days and then in two doses of 50 mg. for the next 9 days. A total of 1250 mg. of acetate, equivalent to 1090 mg. of dehy-

droisoandrosterone, was given. We are greatly indebted to Dr. E. Oppenheimer and Dr. C. R. Scholz of Ciba Pharmaceutical Products, Inc., for the donation of the dehydroisoandrosterone acetate and for the preparation of the solution in oil for parenteral use.

All of the urine was collected during the period of injection and for 2 days after the last injection. The amount of 17-ketosteroids in each daily specimen was determined on an aliquot. The sum of these amounts

TABLE I  
*Summary of Ketonic Fractions Obtained from Chromatographic Column*

Fraction No.	Solvent	Total volume of solvent	Weight of crude fraction	Nature of fraction*
		ml.	mg.	
1	1:1 CCl <sub>4</sub> -petroleum ether	200		Fatty material
2	2:1 " "	125	29	Plates, m.p. 95-97°
3	2:1 " "	100	8	Prisms, " 194-197°
4	4:1 " "	100	Trace	"
5	CCl <sub>4</sub>	100		"
6	" + 0.1% alcohol	200	3	Gum
7	" + 0.2% "	125	21	"
8	" + 0.2% "	75	112	Dehydroisoandrosterone (58 mg.); androsterone (8 mg.)
9	" + 0.2% "	30	48	Androsterone (29 mg.); dehydroisoandrosterone (9 mg.)
10	" + 0.2% "	180	128	Androsterone (88 mg.); dehydroisoandrosterone (12 mg.)
11	" + 0.2% "	75	12	Androsterone (5 mg.) + gum
12	" + 0.3% "	100	25	Etiocholan-3(α)-ol-17-one (14 mg.) + gum
13	" + 0.4% "	250	85	Etiocholan-3(α)-ol-17-one (59 mg.)

\* The weights in parentheses are the weights of the purified substances isolated

was 530 mg. or 49 per cent of the dehydroisoandrosterone administered. The total neutral extract of the hydrolyzed urine, however, assayed 475 mg. of 17-ketosteroids. This discrepancy of 55 mg. may be due to a summation of errors or to a somewhat less efficient extraction of the pooled urine as compared with the aliquots of the daily specimens. The neutral extract was separated into ketonic and non-ketonic fractions with the aid of the Girard-Sandulesco reagent T (5). These fractions were separated further on chromatographic columns of alumina (Merck, standardized according to Brockmann) with appropriate solvents.

Table I summarizes the results obtained with the ketonic material which

was chromatographed by the method of Callow and Callow (1). Fractions 2 and 3 were small in quantity and remain unidentified. In the Zimmermann reaction they gave colors characteristic of the 17-ketosteroids. Fractions 8, 9, and 10 were mixtures of dehydroisoandrosterone and androsterone.

Dehydroisoandrosterone was isolated from Fractions 8 and 9 as the benzoate and from Fraction 10 through the digitonide. The benzoate is well suited to this purpose, since it is sparingly soluble in the common organic solvents. Its high melting point ( $258-260^{\circ}$ ) distinguishes it readily from the benzoates of the other closely associated hydroxysteroids. A total of 79 mg. of dehydroisoandrosterone was accounted for as the benzoate or in the free state.

Androsterone was isolated as the benzoate from Fractions 8 and 9 after removal of the relatively insoluble dehydroisoandrosterone benzoate. It was isolated from Fraction 10 by direct crystallization from acetone and from that fraction in the acetone mother liquor which was not precipitated by digitonin. It was also obtained from Fraction 11 by recrystallization from dilute acetone. The total amount of androsterone accounted for as benzoate or in the free state was 130 mg.

The etiocholan-3( $\alpha$ )-ol-17-one was readily isolated from Fractions 12 and 13 by recrystallization from methanol. The total weight isolated was 73 mg.

Four crystalline non-ketonic fractions were obtained by separation on a chromatographic column of alumina with mixtures of benzene and petroleum ether and of benzene and alcohol. One of these was identified as  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol (6.5 mg.). Another substance (7 mg.) crystallized from dilute methanol as felted needles which melted at  $147-148^{\circ}$ . A third substance (15 mg.) crystallized from methanol as plates which melted at  $143^{\circ}$ . The fourth substance (3 mg.) crystallized from acetone and melted at  $233-234^{\circ}$ . It was not pregnane-3( $\alpha$ ),20( $\alpha$ )-diol.

#### *Comment*

The weights of the purified substances which were isolated are given in Table II. A total of 304 mg. of purified ketosteroids was recovered. This amount is 28 per cent of the 1090 mg. of dehydroisoandrosterone which were administered and 64 per cent of the 475 mg. of 17-ketosteroids which were present in the extract. Less than 1 per cent of the dehydroisoandrosterone administered was recovered as  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol, which was the only non-ketone identified and related to dehydroisoandrosterone.

A striking feature of the results is the relatively large amounts of androsterone and of etiocholan-3( $\alpha$ )-ol-17-one which were isolated. Dehydroisoandrosterone, although an abundant excretory product of patients who

have tumors of the adrenal cortex, in this instance was converted largely to the other two substances before excretion. It constituted only 26 per cent of the total 17-ketosteroids which were isolated. It is not clear from this experiment why dehydroisoandrosterone constitutes 50 to 70 per cent or more of the ketosteroids excreted by many patients who have tumors of the adrenal cortex. However, in these cases there is the possibility that the formation of dehydroisoandrosterone exceeds a maximal rate of disposal of this substance by destruction and by conversion to androsterone and etiocholan-3( $\alpha$ )-ol-17-one and that the excess appears in the urine unchanged.

TABLE II

*Substances Isolated from Urine after Administration of 1250 Mg. of Dehydroisoandrosterone Acetate*

Ketonic fraction		Non-ketonic fraction	
Substance	Weight, purified	Substance	Weight, purified
	mg.		mg.
Dehydroisoandrosterone	79	$\Delta^5$ -Androstene-3( $\beta$ ),17( $\alpha$ )-diol	6.5
Androsterone	130	Felted needles, m.p. 147-148°	7.0
Etiocholan-3( $\alpha$ )-ol-17-one	73	Plates, m.p. 143°	15.0
Plates, m.p. 95-97°	14	Prisms, " 233-234°	3.0
Prisms, " 194-197°	8		

Munson, Gallagher, and Koch (6) examined the urine of a woman who had been given dehydroisoandrosterone acetate parenterally. They isolated dehydroisoandrosterone as the benzoate, in an amount that corresponded to 25 per cent of the conjugated neutral 17-ketosteroids which were extracted by butyl alcohol. The other fractions were not investigated.

The transformation of a 3( $\beta$ )-hydroxysteroid to steroids with a 3( $\alpha$ ) configuration suggests the probability that a 3-ketone was an intermediate in the conversion. If oxidation at C-3 occurred before reduction of the 5,6 double bond,  $\Delta^4$ -androstene-3,17-dione would be the intermediate. This substance is similar to testosterone, which is known to be converted to androsterone and etiocholan-3( $\alpha$ )-ol-17-one (7-10). Conversion of androstenedione to androsterone has been demonstrated also (9) but its conversion to etiocholan-3( $\alpha$ )-ol-17-one has not been shown. If reduction of the 5,6 double bond preceded oxidation at C-3, presumably two intermediates with ketone groups at C-3 would result; namely, androstane-3,17-dione and etiocholan-3,17-dione. These two substances would then yield androsterone and etiocholan-3( $\alpha$ )-ol-17-one respectively on reduction at

C-3. These relations are shown in Fig. 1. The hypothetical intermediates are in brackets. These same compounds have been suggested by Dorfman, Cook, and Hamilton (8) as possible intermediates in the conversion of testosterone to its excretion products.

Although every effort was made to find isoandrosterone, no evidence of its presence either in the free state or as the benzoate could be detected. Almost certainly it was not present in appreciable amounts, although a small amount would be difficult to detect if it were present in the mixtures

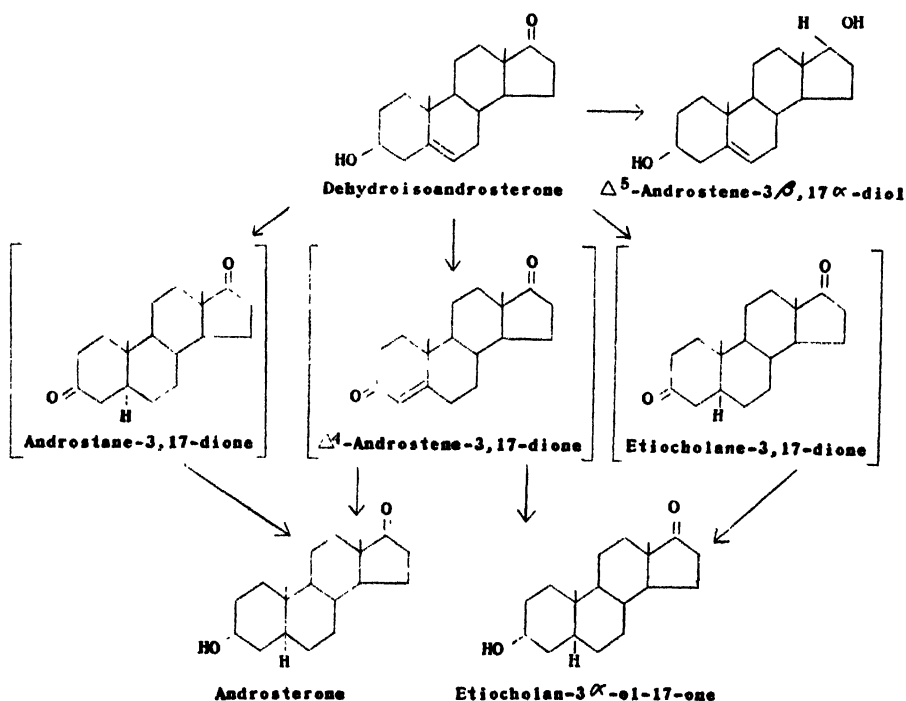


FIG. 1. Steps in the conversion of dehydroisoandrosterone to androsterone and etiocholan-3( $\alpha$ )-ol-17-one.

of androsterone and dehydroisoandrosterone. Isoandrosterone has been isolated from the urine of a man following administration of testosterone (11). If the same intermediates involved in the metabolism of testosterone are also involved in the metabolism of dehydroisoandrosterone, formation of isoandrosterone would be expected.

The isolation of a small amount of  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol is not surprising in view of the isolation of this substance by Hirschmann and Hirschmann (12) from the urine of a boy who had an adrenal cortical tumor. We have also found it in the urine of three women who had adre-



nal tumors and who were excreting relatively large amounts of dehydroisoandrosterone.

Severe anterior pituitary insufficiency probably is accompanied by disturbances which involve the entire organism. The fact that our subject had this condition may have had some influence on the results. However, such disturbances might be expected to result in less metabolism of the dehydroisoandrosterone than in normal individuals instead of resulting in abnormal changes. On the other hand, Munson, Gallagher, and Koch (6) concluded that the greater portion of the dehydroisoandrosterone administered to their patient was not metabolized further but was excreted unchanged. It is planned to continue the study of the metabolism of dehydroisoandrosterone.

So far as could be determined clinically the administration of dehydroisoandrosterone was without effect. The nitrogen balance and the excretion of creatine and creatinine were not influenced.

#### EXPERIMENTAL

*Melting Points*—All of the melting points except one were determined with the Fisher-Johns apparatus. They are recorded as read. The melting point of dehydroisoandrosterone benzoate was determined on a gas-heated block and is corrected for stem exposure.

*Hydrolysis and Extraction of Urine*—The urine was sent to the laboratory at 24 hour intervals. After removal of an aliquot for the determination of the 17-ketosteroids it was acidified with 10 ml. of concentrated HCl and stored in the refrigerator. The urine collected during the first 8 days of the period of injections (18 liters) was concentrated to approximately 3 liters. The concentration of acid which could be titrated with NaOH to Congo red was approximately 0.22 N. The concentrated urine was heated to boiling and enough (95 ml.) concentrated HCl was added to bring the concentration of acid to approximately 0.6 N. Boiling was continued for 20 minutes. The urine was then cooled under the tap and extracted three times with 700 ml. of ether and twice with 500 ml. of chloroform. The pooled specimens collected in the period from the 12th through the 21st day were treated similarly. The respective ether and chloroform extracts were combined. The chloroform extract was reduced to a small volume by distillation and combined with the ether extract which had been concentrated to about 400 ml. The ether solution was washed twice with 50 ml. of 10 per cent NaOH, twice with the same volume of NaOH which contained 1 gm. of sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), and then with water until neutral. After removal of the ether the residue weighed 0.85 gm. and assayed 385 mg. of 17-ketosteroids.

The urine residues were combined and concentrated to 3 liters. The concentration of acid that could be titrated with Congo red as indicator was

increased to normal and the processes of boiling and extraction were repeated. This extract weighed 0.28 gm. and contained 90 mg. of 17-ketosteroids. The total neutral extract was then 1.13 gm. with a content of 475 mg. of 17-ketosteroids.

The neutral extract was separated into ketonic and non-ketonic fractions with the aid of Girard's reagent in the manner described by Wolfe, Fieser, and Friedgood (4).

*Fractionation of Ketonic Material*—The ketonic material (0.63 gm.) was dissolved in 50 ml. of carbon tetrachloride and 50 ml. of petroleum ether (30–60°) were added. A small precipitate was discarded and the solution was passed through a column of 30 gm. of alumina. Various mixtures of carbon tetrachloride and petroleum ether and of carbon tetrachloride with small amounts of alcohol were used in 25 ml. portions for elution. The small fractions so obtained were combined according to their properties to form the major fractions summarized in Table I. Although the concentration of alcohol was increased gradually to 10 per cent by volume, nothing crystalline was obtained after Fraction 13.

*Isolation of Dehydroisoandrosterone and Androsterone*—It will be observed in Table I that a clean separation of dehydroisoandrosterone and androsterone was not achieved. Fraction 8 (112 mg.) was dissolved in 15 ml. of acetone. An equal volume of water was added and the solution was allowed to stand until the acetone had evaporated. In this way 74 mg. of crystalline material were separated from the amorphous material, which remained in a milky suspension. There was some amorphous material present in every fraction even though it appeared to be completely crystalline. Therefore similar treatment was given to the other fractions as a preliminary purification. The 74 mg. of crystals were benzoylated overnight at room temperature with 1 ml. of pyridine and 90 mg. of benzoyl chloride. The pyridine was then largely removed in a stream of air while warmed in a water bath. The residue was taken up in ethyl acetate and washed with dilute HCl, sodium bicarbonate solution, and water. On concentration of the ethyl acetate solution to a small volume 79 mg. of crystals separated. They melted at 256–258° (corrected) and a mixture with authentic dehydroisoandrosterone benzoate (m.p. 259–260°) melted at 256–258° (corrected).

$C_{26}H_{32}O_3$ . Calculated, C 79.55, H 8.22; found, C 79.50, H 8.67

Concentration of the solution to about 0.5 ml. gave another 2 mg. The total amount of benzoate corresponds to 58 mg. of dehydroisoandrosterone. Further concentration of the ethyl acetate solution gave a few crystals, which melted at 210–230°. The ethyl acetate was removed. The residue, crystallized from dilute methanol, yielded two crops of 8 and 3.5 mg. of

crystals, which melted at 170–173°. The melting point was not depressed when these crystals were mixed with androsterone benzoate (m.p. 178–179°).

Fraction 9 yielded 38 mg. of crystals, which melted at 158°. Recrystallization from ethyl acetate failed to change the melting point. Since Fraction 10 had been worked up first, the presence of dehydroisoandrosterone was indicated. Accordingly, 10.0 mg. of this fraction were benzoylated and the product was crystallized from a small amount of ethyl acetate. Only one crop of 3.0 mg. was obtained with this solvent. It melted at 256–257° (corrected) and a mixture with dehydroisoandrosterone benzoate melted at 256–257° (corrected). The ethyl acetate was removed and the residue was crystallized from a little acetone. Three crops amounted to 9.5 mg. and melted at 173–175°, 176–177°, and 170–172° respectively. The melting points were not depressed when these fractions were mixed with androsterone benzoate. The total of 12.5 mg. of the benzoates is equivalent to 9.6 mg. of the alcohols, of which 7.4 mg. or 77 per cent was androsterone. On this basis it was calculated that this fraction contained 29 mg. of androsterone and 9 mg. of dehydroisoandrosterone.

The crystalline portion of Fraction 10 was recrystallized from acetone. Three crops (44 mg.) of androsterone, m.p. 182–183°, were obtained.

$C_{19}H_{30}O_2$ . Calculated, C 78.57, H 10.41; found, C 78.37, H 10.49

There was no depression of the melting point when this fraction was mixed with authentic androsterone.

The acetate, prepared with pyridine and acetic anhydride, melted at 163–165°.

$C_{21}H_{32}O_3$ . Calculated, C 75.86, H 9.70; found, C 75.94, H 9.98

The fourth crop obtained from acetone melted at 152–153° and gave a precipitate with digitonin. This crop and the remainder in the mother liquor were combined in 5 ml. of 90 per cent methanol; 300 mg. of digitonin in 15 ml. of hot 90 per cent methanol were added. Since there was no precipitate after standing overnight, the water content was increased to 30 per cent. After standing in the refrigerator 70 mg. of digitonide separated. It was decomposed with pyridine and ether and yielded 12 mg. of material which melted at 134–136°. It was converted to the benzoate, which melted at 251–256° (corrected), and was therefore taken to be dehydroisoandrosterone benzoate. The portion which was not precipitated with digitonin yielded 44 mg. of androsterone, which melted at 182–183°. Fraction 10 was thus separated into 88 mg. of androsterone and 12 mg. of dehydroisoandrosterone.

Fraction 11 was recrystallized from dilute acetone and 5 mg. of crys-

tals were obtained. The melting point of 178–180° suffered no depression when the crystals were mixed with androsterone.

*Isolation of Etiocholan-3( $\alpha$ )-ol-17-one*—This substance was isolated from Fractions 12 and 13 by crystallization from methanol. From the former 14 mg. of crystals with a melting point of 143–145° were obtained. The first crop of Fraction 13 melted at 150–151° with a transition point at 138–140°. A mixture with a specimen of etiocholan-3( $\alpha$ )-ol-17-one (m.p. 150–151°) also melted at 150–151°. The benzoate prepared with benzoyl chloride and pyridine melted at 161–162° and its melting point was not depressed when it was mixed with an authentic specimen of etiocholan-3( $\alpha$ )-ol-17-one benzoate.

$C_{26}H_{34}O_3$ . Calculated, C 79.15, H 8.69; found, C 78.61, H 9.00

*Ketonic Fraction 2*—This fraction crystallized from dilute acetone as large plates, which melted at 95–97°. Repeated crystallization failed to change this melting point.

*Ketonic Fraction 3*—This fraction was very small. After crystallization from dilute acetone it melted at 176–180°. A few prisms were obtained by recrystallization from acetone. They melted at 194–197° for the most part, although a few crystals melted sharply at 203°.

Small amounts of ketonic Fractions 2 and 3 were dissolved in 3 drops of absolute alcohol. 3 drops each of a 2 per cent solution of *m*-dinitrobenzene and of 2.5  $\times$  potassium hydroxide in absolute alcohol were added. The red color characteristic of the 17-ketosteroids quickly developed.

*Fractionation of Non-Ketonic Fraction*—Although this fraction weighed 440 mg., the weight of crystalline material that could be obtained was only 31.5 mg. after purification. The fraction was extracted with a total of 30 ml. of hot benzene. This left undissolved 56 mg. of highly colored amorphous material which was discarded. Addition of 30 ml. of petroleum ether (30–60°) precipitated 41 mg. of similar material, which also was discarded. The resulting solution was passed through a column of 16 gm. of alumina. The first crystalline fraction, 7 mg., was eluted with a mixture of benzene and petroleum ether. It separated as felted needles from dilute methanol and melted at 147–148°. Immediately following this fraction, when the solvent was changed to a 2:1 mixture of benzene and petroleum ether, was a fraction of 15 mg. which crystallized from methanol as plates with a melting point of 143°. As the solvent was changed to benzene and to benzene with small amounts of alcohol, only traces of oil were removed from the column until benzene containing 0.2 per cent of alcohol was reached. This mixture removed 26 mg. (crude weight) of crystalline material.

*Isolation of  $\Delta^5$ -Androstene-3( $\beta$ ),17( $\alpha$ )-diol*—The fraction which was re-

moved with 0.2 per cent alcohol in benzene was crystallized from methanol. The first crop, 2.5 mg., melted at 173–174°. A mixture with  $\Delta^5$ -androsterone-3( $\beta$ ),17( $\alpha$ )-diol (m.p. 177–178°), prepared from dehydroisoandrosterone (13), melted at 177–178°. The acetate was prepared from the second and third crops (4.0 mg.) with acetic anhydride and pyridine. It crystallized from methanol as plates which melted at 159–160°. A mixture with  $\Delta^5$ -androsterone-3( $\beta$ ),17( $\alpha$ )-diol diacetate (m.p. 159–160°) melted at 159–160°.

The last fraction from which crystals were obtained was removed with 0.5 per cent alcohol in benzene. The crystals were freed from much gum by recrystallization from acetone but with considerable loss. The first crop (2 mg.) melted at 233–234°. The second crop of about 1 mg. melted at 208–215°. A mixture of the first crop with pregnane-3( $\alpha$ ),20( $\alpha$ )-diol melted at 210–215°.

#### SUMMARY

Following the administration of 1090 mg. of dehydroisoandrosterone (as the acetate) to a man who had anterior pituitary insufficiency, 79 mg. of dehydroisoandrosterone, 130 mg. of androsterone, 73 mg. of etiocholan-3( $\alpha$ )-ol-17-one, and 6.5 mg. of  $\Delta^5$ -androsterone-3( $\beta$ ), 17( $\alpha$ )-diol were recovered from the urine. Two other crystalline ketones and four non-ketones were obtained in small amounts but not identified.

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# STUDIES ON THE FORMATION OF FOLIC ACID BY INCUBATING *LACTOBACILLUS CASEI* FACTOR AND PYRACIN WITH CHICK LIVER\*

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In 1940 Snell and Peterson (1) announced the discovery of a factor, necessary for the growth of *Lactobacillus casei*, which they called the "norit eluate factor." Hutchings, Bohonos, and Peterson (2) the following year reported that concentrates containing this factor were also active for the growth of *Streptococcus lactis* R.<sup>1</sup> At about the same time Mitchell, Snell, and Williams (4) obtained from spinach a concentrate which was highly active for *Streptococcus lactis* R and also required by *Lactobacillus casei*. They named the active substance folic acid.

Pfiffner and associates (5), in 1943, reported the isolation in crystalline form of an antianemic factor for chicks which was active in promoting the growth of both *Lactobacillus casei* and *Streptococcus lactis* R. Because of similarities in biological and physical properties they considered it to be identical with Hogan and Parrott's (6) vitamin B<sub>c</sub> and also suggested that vitamin B<sub>c</sub> was probably the same as folic acid and the "norit eluate factor."

The same year Stokstad (7) described two *Lactobacillus casei* factors, one from liver and the other from yeast. These were believed not to be identical, since the compound from liver was equally active for both organisms while that from yeast was only half as active for *Streptococcus lactis* R as for *L. casei*. Since the chemical analysis of the compound from liver agreed with that reported for vitamin B<sub>c</sub> by Pfiffner and associates (5), Stokstad concluded that his liver *L. casei* factor was probably identical with vitamin B<sub>c</sub>.

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<sup>1</sup> Niven and associates (3) have found that the organism generally known as *Streptococcus lactis* R is the enterococcus, *S. faecalis*. This organism is carried by the American Type Culture Collection as No. 8043. Since the *S. lactis* R factor is one of the growth factors involved, we have continued in this paper to call the organism *S. lactis* R to prevent confusion.

Binkley and associates (8), on the other hand, reported that vitamin B<sub>9</sub>, isolated from enzyme-digested yeast by a procedure similar to that used in isolating this substance from liver, had the same activity for *Lactobacillus casei* and *Streptococcus lactis* R as had vitamin B<sub>9</sub> isolated from liver. In addition, the properties of the two crystalline compounds as well as the chemical analyses were the same; so the authors concluded that the compounds from yeast and liver were identical. Krueger and Peterson (9) have also found no difference in potency between the vitamin B<sub>9</sub> preparation from liver and that from yeast.

Recently Hutchings, Stokstad, Bohonos, and Slobodkin (10) reported the isolation of a third *Lactobacillus casei* factor which possessed 85 to 90 per cent as much activity for *L. casei* as did Stokstad's (7) *L. casei* factor from liver, yet had only 6 per cent of the liver *L. casei* factor activity when assayed with *Streptococcus lactis* R. By our method of analysis we have found the activity of this factor for *Streptococcus lactis* R to be approximately 2.5 per cent that of the *L. casei* activity.

Keresztesy, Rickes, and Stokes (11) have announced another factor from an unstated source, designated by them as the *Streptococcus lactis* R factor, which is active for *S. lactis* R but inactive for *Lactobacillus casei*. Stokes, Keresztesy, and Foster (12) found that, when *S. lactis* R was allowed to grow on a medium containing the *S. lactis* R factor, a substance was produced in the medium which was active for *L. casei*. Day, Mims, Totter, Stokstad, Hutchings, and Sloane (13) found that by treating a highly purified preparation of *L. casei* factor with the rat liver enzyme solution of Mims, Totter, and Day (14) the activity for *S. lactis* R was greatly increased.

These active factors fall into three general classes, those that are active for *Lactobacillus casei* but possess little activity for *Streptococcus lactis* R, those active only for *S. lactis* R, and those that are active for both organisms. As a matter of convenience and to simplify terminology, we shall henceforth refer to all factors that are active mainly for *L. casei* as the *L. casei* factor, those active for *S. lactis* R only as the *S. lactis* R factor, and those active for both organisms as folic acid.

Using the new *Lactobacillus casei* factor of Hutchings and associates (10), Scott, Norris, Heuser, and Bruce (15) have shown that either  $\alpha$ - or  $\beta$ -pyracin lactone<sup>2</sup> is required besides *L. casei* factor for the prevention of anemia in chicks. Pfiffner and associates (5) and others, on the other hand, have

<sup>2</sup> Pyracin refers to 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine ( $\alpha$ -pyracin), or 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine ( $\beta$ -pyracin). The lactones of these acids have recently been named  $\alpha$ - and  $\beta$ -pyracin by Scott and associates (15). Since unpublished data show that the acid form is also active nutritionally, the name pyracin, because of its connotation, will henceforth refer to the acid form, and the lactones will be designated as  $\alpha$ -pyracin lactone and  $\beta$ -pyracin lactone. Huff and Perlzweig (16) previously called  $\beta$ -pyracin, pyridoxic acid.

reported that pure crystalline compounds which are active for both *L. casei* and *Streptococcus lactis* R are effective in preventing this anemia.

In the course of an investigation on the relationship between *Lactobacillus casei* factor, pyracin, and folic acid by means of incubation studies with fresh chick liver, results were obtained which furnish an explanation of the apparent discrepancy between the work of Scott and associates (15) and the work of Piffner and associates (5) and others. These results are presented in this report.

#### EXPERIMENTAL

*Livers for Incubation*.—The livers used in the study reported in this paper were obtained from white Leghorn cockerels, 8 to 13 weeks of age. From hatching to the 8th week the chicks were fed a diet known to be partially deficient in unidentified factors present in liver and yeast. At 8 weeks of age the diet was changed to a commercial chick starter of known formula.

*Incubation Procedure* In general, the incubation procedures of Wright and Welch (17) and Mims, Totter, and Day (14) were followed. 1 part of fresh liver was placed in a Waring blender with 5 parts of 0.05 M phosphate buffer at pH 7.0. After 4 minutes of blending, the mixture was centrifuged for 20 minutes, and the portion remaining suspended in the phosphate buffer was decanted into a flask and made to a suitable volume. The samples were incubated under toluene overnight at 37°, after which they were steamed for 0.5 hour, diluted to 500 ml., and filtered through Whatman No. 44 filter paper with Celite Filter-Aid.

*Folic Acid Determinations*.—The samples were assayed for folic acid by the method of Luckey, Briggs, and Elvehjem (18), with *Streptococcus lactis* R as the test organism. The medium was modified to include 100 millimicrograms of pyridoxamine<sup>3</sup> per tube. The samples were run in duplicate at two levels. Williams' 7.7 per cent folic acid<sup>4</sup> concentrate and Stokstad's liver folic acid<sup>5</sup> were used as standards at the levels of 10, 20, 40, 60, 80, and 1, 2, 3, 4, 5 millimicrograms per tube, respectively.

*Incubation of Lactobacillus casei Factor with Pyracin*.—Flasks containing liver suspensions equivalent to 62.5 mg. of original liver, 50  $\gamma$  of  $\alpha$ - or  $\beta$ -pyracin,<sup>6</sup> and 1  $\gamma$  of *Lactobacillus casei* factor separately and in all possible com-

<sup>3</sup> We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the pyridoxamine and pyracin lactones.

<sup>4</sup> We are indebted to Dr. R. J. Williams of the University of Texas, Austin, Texas, for the folic acid concentrate.

<sup>5</sup> We are indebted to Dr. E. L. R. Stokstad and Dr. B. L. Hutchings of the Lederle Laboratories, Inc., Pearl River, New York, for the liver folic acid and for the two samples of crystalline *Lactobacillus casei* factor.

<sup>6</sup> The pyracin was prepared by refluxing for 15 minutes 5 mg. of pyracin lactone in 50 ml. of H<sub>2</sub>O to which had been added 20 drops of 1 N NaOH. After cooling, the solution was made to pH 6.8 to 7.0 by the addition of 1 N HCl.



binations were taken through the incubation procedure and assayed for folic acid. Five experiments were run, with different livers each time and freshly prepared pyracin solutions. The first three experiments were conducted with one sample of *L. casei* factor (Sample J-56) and the last two with a second one (Sample L-84-12). When not incubated with liver both the samples of *L. casei* factor and pyracin, alone and in combination, were inactive for *Streptococcus lactis* R at the levels used in these studies. The results are presented in Table I.

TABLE I  
*Synthesis of Folic Acid by Incubating Lactobacillus casei* Factor and Pyracin  
with Chick Liver

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5		Average
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(b)
Chick liver . . . . .	6.16		9.74		1.64		4.28		0.16		
“ “ + <i>L. casei</i> factor . . . . .	11.80	0.35	13.24	0.22	11.46	0.61	7.72	0.22	1.64	0.19	0.32
Chick liver + $\alpha$ -pyracin . . . . .	7.84		9.46		3.76		6.82				
“ “ + $\beta$ -pyracin . . . . .	7.72		9.54		3.30		6.52		1.75		
“ “ + <i>L. casei</i> factor + $\alpha$ -pyracin . . . . .	16.20	0.63	21.60	0.74	14.70	0.82	11.09	0.43			0.66
Chick liver + <i>L. casei</i> factor + $\beta$ -pyracin . . . . .	16.80	0.67	22.18	0.78	14.04	0.78	11.40	0.45	4.55	0.55	0.65

(a) Micrograms of folic acid per gm. of liver. (Stokstad's crystalline liver folic acid, active for *Streptococcus lactis* R and *Lactobacillus casei* as standard.) (b) Micrograms of folic acid formed per microgram of *Lactobacillus casei* factor used. (Stokstad's crystalline *Lactobacillus casei* factor inactive for *Streptococcus lactis* R at the levels used.)

The results show that under the experimental conditions *Lactobacillus casei* factor was converted into some substance that is active for *Streptococcus lactis* R. Pyracin incubated alone with liver usually gave some increase in folic acid content, but when incubated with *L. casei* factor and liver caused a marked increase in response. The increase in folic acid produced by incubating liver, *L. casei* factor, and pyracin was greater than the sum of the increases caused by incubating liver and *L. casei* factor alone and liver and pyracin alone.

When *Lactobacillus casei* factor was incubated with chick liver, 0.32  $\gamma$  of folic acid was produced per microgram of *L. casei* factor used. Incubating both pyracin and *L. casei* factor with liver caused approximately a 100 per cent increase in folic acid over that formed by *L. casei* factor alone.

*Incubation of Lactobacillus casei* Factor with Pyracin Lactones—Freshly

prepared solutions of the pyracin lactones were incubated with liver and *Lactobacillus casei* factor. The addition of the lactones to the liver and *L. casei* factor mixture caused an inhibition in the conversion of *L. casei* factor to folic acid. On the other hand, when solutions of the lactones which had been refrigerated for 3 days or more were used, results similar to those obtained with the free acids were secured. The results of these studies are given in Tables II and III.

Since it is known that lactones of this type are unstable in solution near neutrality, the probable explanation of the conflicting results obtained with the fresh and stored lactone solutions is that hydrolysis of the lactone oc-

TABLE II

*Inhibition of Folic Acid Synthesis by Incubating Lactobacillus casei Factor and Fresh Solutions of Pyracin Lactones with Chick Liver*

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Average
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	
Chick liver	1.64		5.12		4.58		7.92		
“ “ + <i>L. casei</i> factor.	11.46	0.61	14.24	0.56	10.16	0.35	11.02	0.20	0.43
“ “ + $\alpha$ -pyracin lactone	3.28		4.66		6.48		8.40		
Chick liver + $\beta$ -pyracin lactone	2.80		4.26		5.64		6.06		
Chick liver + <i>L. casei</i> factor + $\alpha$ -pyracin lactone	8.60	0.44	9.88	0.30	8.84	0.27	8.84	0.06	0.27
Chick liver + <i>L. casei</i> factor + $\beta$ -pyracin lactone	6.88	0.33	12.46	0.46	9.80	0.33	7.50	-0.03	0.27

(a) Micrograms of folic acid per gm. of liver. (b) Micrograms of folic acid formed per microgram of *Lactobacillus casei* factor used.

curred and an equilibrium was reached in which sufficient acid was present in the mixture to bring about the observed results. Because the acid form fluoresces very slightly at pH 9.0 (16), while the lactone form is intensely fluorescent at this pH, it was possible to substantiate this hypothesis by measuring the fluorescence of  $\beta$ -pyracin lactone at varying times following the preparation of a fresh solution. The lactone was kept in a phosphate buffer at pH 7.0 in the refrigerator, and aliquots were taken daily for the fluorescence study. The fluorescence of a phosphate buffer solution at pH 9.0 containing 5 millimicrograms of  $\beta$ -pyracin lactone per ml. was determined by using a Coleman model 12 electric photofluorometer with Filters B-1 and PC-1. The measurements were made immediately and daily for 7 days. The galvanometer readings fell from 83.5 to a constant value of 37.5 in this time, the equilibrium being reached on the 3rd day. The equilibrium mixture was found to contain 45 per cent of the lactone.

The free acid appeared, therefore, to be the substance involved in producing the increase in folic acid obtained in these experiments. The decrease in the amount of folic acid formed when newly prepared lactone solutions and *Lactobacillus casei* factor were incubated with liver, as compared to the increase in folic acid when *Lactobacillus casei* factor was incubated alone, indicates inhibition due to the formation of an antifolic acid or an antienzyme.

TABLE III

*Synthesis of Folic Acid by Incubating Lactobacillus casei Factor and Old Solutions\* of Pyracin Lactones with Chick Liver*

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Average
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	
Chick liver . . . . .	9.74		5.12		4.58		7.92		
“ “ + <i>L. casei</i> factor . . . . .	13.24	0.22	14.24	0.56	10.16	0.35	11.02	0.20	0.33
“ “ + $\alpha$ -pyracin lactone . . . . .	10.02		6.58		6.37		8.36		
Chick liver + $\beta$ -pyracin lactone . . . . .	10.74		5.23		6.64		8.72		
Chick liver + <i>L. casei</i> factor + $\alpha$ -pyracin lactone . . . . .	19.40	0.60	16.50	0.71	15.45	0.68	13.48	0.35	0.59
Chick liver + <i>L. casei</i> factor + $\beta$ -pyracin lactone . . . . .	17.40	0.48	15.40	0.64	13.85	0.58	12.70	0.30	0.50

(a) Micrograms of folic acid per gm. of liver. (b) Micrograms of folic acid formed per microgram of *Lactobacillus casei* factor used.

\* Used after holding 3 or more days in the refrigerator.

#### DISCUSSION

The data presented in this paper demonstrate that chick liver when incubated with *Lactobacillus casei* factor brings about the same marked increase in *Streptococcus lactis* R activity as was observed by Day and associates (13) with rat liver. Furthermore, it is evident that pyracin is concerned in the production of folic acid from *L. casei* factor by chick liver. The mechanism through which pyracin brings about this change is not clear. It is possible that pyracin combines with *L. casei* factor to form folic acid, or that pyracin may be involved in an enzyme system concerned in the formation of folic acid from *L. casei* factor.

The inhibition of the folic acid synthesis produced by the pyracin lactones can be explained by either of these hypotheses. In the first instance, conjugation between *L. casei* factor and the lactones could form an antifolic acid, and in the second place, an antienzyme could be produced. Further studies are in progress to determine the details of this mechanism.

## SUMMARY

1. The incubation of *Lactobacillus casei* factor with fresh chick liver caused a marked increase in the folic acid content as measured by *Streptococcus lactis* R.

2. When both *Lactobacillus casei* factor and pyracin were incubated with liver, the increase in folic acid was approximately twice as great as that obtained by incubating *Lactobacillus casei* factor alone with liver.

3. The pyracin lactones inhibited the conversion of *Lactobacillus casei* factor to folic acid when incubated with liver.

4. The action of pyracin in promoting an increased production of folic acid may be explained by conjugation with *Lactobacillus casei* factor to form folic acid, or by entering into an enzyme system that brings about the conversion of *Lactobacillus casei* factor to folic acid.

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# EFFECT OF DIFFERENT POLYSACCHARIDES AND POLYSACCHARIDE DEGRADATION PRODUCTS ON THE ACTIVITY OF POTATO PHOSPHORYLASE\*

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A synthetic polysaccharide has been obtained from glucose-1-phosphate by the action of a phosphorylase from muscle (1) or one from potatoes (2, 3). Both enzymes appear to produce the same polysaccharide (4). The polysaccharide has been termed a synthetic "starch," because a similar, if not identical, product can be obtained by fractionation of natural starches by means of butanol, isoamyl alcohol, or other polar solvents (5). This fraction, which is generally called amylose (6), appears to be made up of long unbranched chains of glucopyranose units in 1,4- $\alpha$ -glucosidic linkages (4).

Studies on the enzymatic synthesis showed that it was necessary to activate the phosphorylases by having present small amounts of soluble starch or glycogen (1-3). Obviously, it is important to understand the rôle of such activator carbohydrates in polysaccharide synthesis. Synthetic amylose polysaccharides have been regarded as incapable of activating either potato (2, 3) or muscle (4, 7) phosphorylase. Various natural starches, glycogen, and some dextrans have activating ability for the potato enzyme (3, 8). The Cori (7) group believes that, "Polysaccharide synthesis [by muscle phosphorylase] might consist in a lengthening of existing side chains [of glycogen or natural starch] by addition of glucose units in 1:4 glucosidic linkages." The apparent inability of amyloses to act as activators is attributed to the low concentration of end-groups and low solubility. Since the polysaccharides formed by the enzymes of potatoes and muscle are so similar, it might be assumed that the potato phosphorylase also requires branched chain activators of the amylopectin type.

The purpose of the present investigation was to determine the effect of partial acid hydrolysis, and certain other factors, on the ability of

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different polysaccharides to activate potato phosphorylase. Preliminary to this, however, it seemed advisable to investigate the preparation of suitable enzyme concentrates and the determination of enzyme activity. Therefore, the results of such studies are also given here. Some of the data obtained in this investigation have been reported in a preliminary note (9).

#### EXPERIMENTAL

*Materials*—All the glucose-1-phosphate was prepared by the method of Hanes (2) and recrystallized until the  $[\alpha]_D^{20}$  of the dipotassium salt dihydrate was 75–79°.

Potato phosphorylase was purified by fractional precipitation with ammonium sulfate. A sufficient quantity of potatoes (*Solanum tuberosum*) was peeled and sliced to furnish at least 1 liter of juice. They were then kept under water until used, usually within 1 hour. Then they were drained quickly and pulped in a Waring blender. The crude juice was brought to a specific gravity of 1.085 with solid ammonium sulfate and centrifuged within  $\frac{1}{2}$  hour. The supernatant liquid was raised to a specific gravity of 1.152 with ammonium sulfate and then recentrifuged. The precipitate, which was not entirely soluble in water, was suspended in 250 ml. of water and fractionation was repeated in a specific gravity range of 1.095 to 1.145. The precipitated enzyme was dissolved in 100 ml. of water for two subsequent fractionations in the specific gravity ranges of 1.100 to 1.140 and 1.100 to 1.135 respectively. The pH was determined at frequent intervals throughout the preparation and was kept between 6.0 and 6.5 by the addition of dilute ammonium hydroxide. Inactivation of the enzyme is rapid below pH 5.8.

To preserve the final enzyme precipitate for subsequent use it was generally taken up in water and diluted to 25 ml. for each liter of crude potato juice used. The dark solution was stored at 1–2°. No suitable preservative was found. Merthiolate (Lilly) and thymol caused marked inactivation. Owing to the occasional need for maintenance of the enzyme at water bath temperatures for considerable periods of time, the stability of the most concentrated preparations was studied at 38°. At pH 6.2 no appreciable decrease in activity occurred for 32 minutes. The activity scarcely changes for 4 to 6 weeks when stored at 1–2°.

Experience has shown that one of the most important precautions in the concentration of the enzyme is a speedy separation of the freshly expressed juice from the pulp. Inactivation is particularly rapid at this stage. All other steps in the procedure should be carried out as fast as practicable.

An attempt to prepare colorless concentrates was made with *p*-amino-

benzoic acid and sulfanilamide to inhibit tyrosinase activity in the juice (10). Although *p*-aminobenzoic acid was partially effective, it was not regarded as satisfactory enough to warrant its use in this work. Sulfanilamide had no effect.

Nitrogen was determined on aliquots of the various fractions by the micro-Kjeldahl method. Protein was precipitated in a 15 ml. Pyrex centrifuge tube with trichloroacetic acid and washed three times, by centrifugation, to remove ammonium sulfate. Acid digestion was carried out in the tube. The purest preparations showed an activity of 18.2 units per mg. of nitrogen. This appears to be almost identical with the activity of Green and Stumpf's (3) most active preparations.

Repeated preparations of the enzyme concentrate by this means have demonstrated good reproducibility of results, an accomplishment difficult to achieve when the quantity of ammonium sulfate to be added in each fractionation is determined by the degree of saturation. Therefore, this method of concentration seems to be more satisfactory than the customary procedure (3) which is based on the addition of ammonium sulfate to specified degrees of saturation.

Synthetic polysaccharide was prepared by incubating about 280 units (as determined by our method) of purified potato phosphorylase in 0.25 M citrate buffer at pH 6.2 with 12.5 gm. of dipotassium glucose-1-phosphate dihydrate at 37°. The total volume of the reaction mixture was about 250 ml. The insoluble polysaccharide that formed was centrifuged off after about 24 hours, washed several times with water and then with alcohol, and dried at 70°. The yield was 3.2 gm. or 62.5 per cent of the quantity theoretically possible at that pH. The nitrogen content could not have exceeded 0.48 per cent, because the added phosphorylase contained only 15.4 mg. of nitrogen.

The linear fraction of corn-starch (amylose) was prepared according to the method of Schoch (5).

*Modification of Green and Stumpf's Method for Determination of Phosphorylase Activity*—According to Green and Stumpf (3), the activity of potato phosphorylase is directly proportional to the concentration of the enzyme even when the activity is great enough to liberate approximately 1 mg. of inorganic phosphorus per 3 minutes. When the reaction is allowed to run 5 to 10 minutes, as recommended for their method, the total amount of phosphorus liberated will be, by calculation, about 1.7 to 3.3 mg., if the reaction is strictly linear under such conditions. Since the total amount of phosphorus furnished by the glucose-1-phosphate used in their determinations is only 3.1 mg., and at equilibrium not more than 80 per cent can be inorganic, it is obvious that the permissible enzyme concentration must be more precisely defined.



Figs. 1 to 4<sup>1</sup> show the effects of enzyme concentration, temperature, added polysaccharide, and glucose-1-phosphate concentration on the

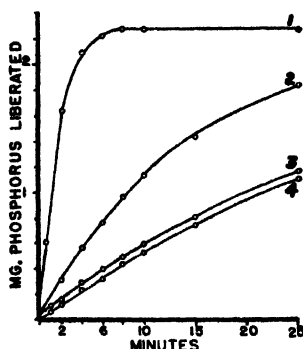


FIG. 1

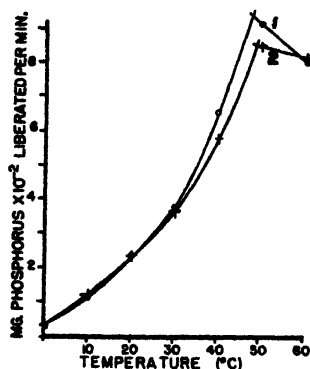


FIG. 2

FIG. 1. Rate of phosphorus liberation per 3.5 ml. of reaction mixture with varying concentrations of purified phosphorylase. Curve 1, concentrated enzyme solution; Curve 2, 1:10 dilution of concentrated solution; Curve 3, 1:25 dilution; Curve 4, 1:25 dilution less blank.

FIG. 2. Effect of temperature on rate of phosphorus liberation by purified phosphorylase. Curve 1, incubation period of 5 minutes; Curve 2, incubation period of 10 minutes.

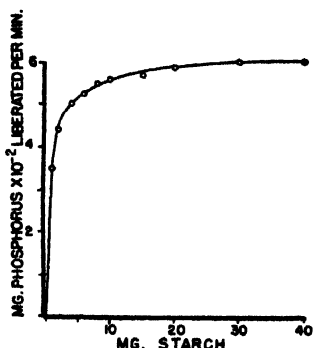


FIG. 3

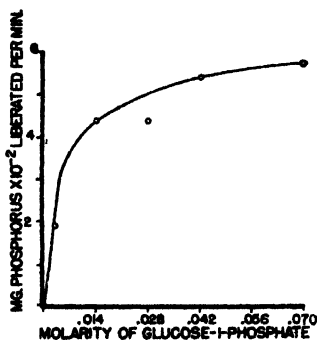


FIG. 4

FIG. 3. Effect of added polysaccharide activator on phosphorylase activity (soluble starch, Merck).

FIG. 4. Effect of glucose-1-phosphate on activity of phosphorylase. The abscissa represents the concentration in the reaction mixture.

rate of phosphate liberation. These data demonstrate that the activity is not directly proportional to the enzyme concentration when the latter is great enough to cause the liberation of much more than 1.0 mg. of phos-

<sup>1</sup> Data necessary for the preparation of Fig. 4 were obtained by Miss Elsa Proehl.

phorus per 10 minutes (Fig. 1). Also, it is worthy of note that the temperature coefficient is approximately 2.0 at the temperature interval 10–20° and it approaches 1.3 at 40–50° (Fig. 2). Thus the enzyme behaves like many others in this respect (11). Green and Stumpf's (3) finding that 10 to 20 mg. of soluble starch provide for maximal activation of the enzyme was confirmed (Fig. 3). As shown in Fig. 5, there is no autocatalytic action in the synthesis of polysaccharide from glucose-1-phosphate by potato phosphorylase.

On the basis of these data we have used as the unit of phosphorylase activity that amount of enzyme which will liberate 0.1 mg. of inorganic phosphorus in 3 minutes from glucose-1-phosphate at 38° and pH 6.2. The reaction mixture is composed of 1.0 ml. of enzyme solution of such dilution that not more than 0.5 mg. of phosphorus will be liberated in

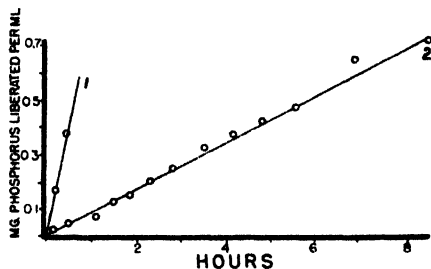


FIG. 5. Rate of phosphorus liberation by purified phosphorylase. Curve 1, in the presence of 20 mg. of soluble starch per 3.5 ml. of reaction mixture; Curve 2, in the absence of added polysaccharide.

10 minutes, 0.5 ml. of 1.0 M citrate buffer of pH 6.2, 1.0 ml. of 2 per cent soluble starch solution; 1.0 ml. of 0.1 M glucose-1-phosphate is added to the rest of the mixture after both have been brought to temperature equilibrium. The period of incubation is 10 minutes. The reaction is halted by rapid addition of 2.5 ml. of 10 per cent trichloroacetic acid, and phosphorus is determined on an aliquot of the reaction mixture by the Fiske and Subbarow method (12).

Extraction of the reactants with diphenylthiocarbazone (dithizone) in carbon tetrachloride at pH 6.2 had no effect on the activity of the enzyme. Also, manganous chloride, cobaltous sulfate, nickelous sulfate, thiamine, urea, *p*-aminobenzoic acid, and sulfanilamide, in moderate concentrations, were without effect. Thus it appears that the concentration of various heavy metals and certain common organic substances is not of importance in phosphorylase determinations, unless extraordinary quantities are present.

*Effect of Acid Hydrolysis on Ability of Different Polysaccharides to Activate Phosphorylase*—It was reasoned that the reported inability of synthetic polysaccharide to act as an activator might be due to structural arrangements causing an inadequate concentration of groupings necessary for that function. It seemed likely that partial acid hydrolysis of the synthetic material, or dispersion with alkali, might produce active substances.

A typical experiment with acid is described. After correcting for ash, 2.5 gm. of starch were suspended in 18 ml. of water in a 50 ml. volumetric flask. The flask and contents were chilled and then concentrated hydrochloric acid, also chilled, was added to the mark. The mixture was agitated continuously until all the polysaccharide was in solution. This required 3 to 4 minutes. The temperature was kept at 27°. At the end of 5 minutes the first sample, 1.0 ml., was removed and transferred to a 10 ml. volumetric flask in which had been placed nearly enough 1.65 N sodium hydroxide to neutralize the acid. By adding small amounts of sodium hydroxide the neutralization was quickly completed and water was added to the mark. Constancy of pH in subsequent neutralized samples was assured by transferring 1.0 ml. aliquots (equivalent to 5 mg. of starch) to test-tubes, each containing 0.5 ml. of the citrate buffer at pH 6.2. The activating power was determined as described earlier, with the neutralized samples in place of soluble starch in the test mixtures.

As indicated in Figs. 6 and 7 the activating ability of the hydrolyzing polysaccharide solutions increased progressively to a maximum and then gradually declined. The reducing value (copper) of the solutions became maximal after all activating ability had been lost. The iodine-staining reaction of the hydrolysates disappeared by the 4th to 9th hour. The achromic point was reached quickest by hydrolyzing corn-starch and slowest by the linear component of corn-starch (amylose). The activating ability began to decline almost simultaneously with disappearance of the iodine color reaction. Potato and arrowroot starches, when similarly treated, gave almost identical results to corn-starch (Fig. 6).

Higher concentrations of acid caused the maximal activating effect to be reached sooner, and, in other respects also, the effects on the polysaccharides were telescoped. This is shown by Fig. 7 which summarizes an experiment in which the normality of the acid solution was increased to 10.1. All other conditions were the same as previously described. Under such conditions the achromic point was reached in less than 2 hours and the activating ability passed from a high value to almost zero in less than 5 hours.

In some other experiments the partially hydrolyzed activators were tested in concentrations of 0.5 mg. per determination. The principal results have been reported briefly (9). They show that each substance

attains approximately the same level of activating power, but it requires a shorter period of hydrolysis for the corn-starch to reach maximal activity than the essentially pure amyloses, "synthetic" potato starch, and Schoch's butanol-insoluble fraction of corn-starch.

The data indicate that activation of potato phosphorylase is not dependent upon branched chain polysaccharides, because the partial hydrolysis of synthetic polysaccharide and the butanol-insoluble fraction of corn-starch yields activators as effective as similarly treated natural starches that are rich in branched chain glucose units. Also, the results with achroodextrins suggest that the carbohydrate activator of potato

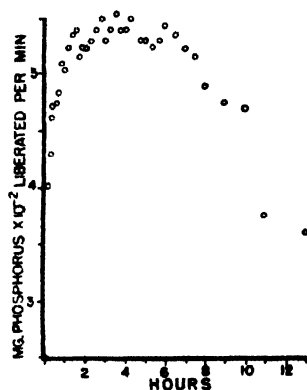


FIG. 6

FIG. 6. Effect of hydrolysis by 7.2 N HCl on the activating power of corn-starch. The abscissa represents the length of hydrolysis of the polysaccharide; the ordinate, the rate of phosphorus liberation in the presence of 5 mg. of hydrolyzed polysaccharide.

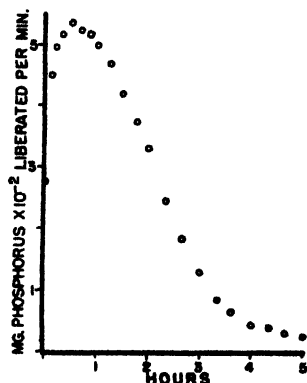


FIG. 7

FIG. 7. Same as Fig. 6, except that 10.1 N HCl is used.

phosphorylase need not contain more than 7 or 8 glucose residues per molecule, because Hanes and Cattle (13) have reported that polysaccharides may be fragmented to dextrins which appear to contain only 7 to 8 glucose units before giving an achromic iodine test. Thus it seems probable that purer synthetic amylose could be obtained by means of partially acid-hydrolyzed amylose as the activator of potato phosphorylase. The preparations made by present methods are impure (4).

Although the conditions employed for acid hydrolysis may cause the condensation of some glucose into branched chain structures (14) it does not seem possible that this could account for the enzyme activation, because the latter effect subsides and eventually disappears, whereas the condensation products remain.

*Effect of Alkali on Activating Ability of Synthetic Potato Polysaccharide*—To 50 mg. of the synthetic material suspended in about 5 ml. of water 1 drop of concentrated potassium hydroxide was added. The polysaccharide dissolved almost immediately. The solution was quickly neutralized with hydrochloric acid and diluted to 10.0 ml. The activating ability was found to be within the range of corn-starch hydrolyzed with hydrochloric acid for 10 minutes (Table I). After 48 hours extensive retrogradation had occurred and the supernatant solution was scarcely more active than a saturated aqueous solution of untreated synthetic polysaccharide.

*Degree of Acid Hydrolysis of Activating Polysaccharide and Iodine Color Reaction of Synthesized Products*—The unexpected results of a lecture demonstration led us to consider the effect of the activator polysaccharide on the nature of the synthesized product as indicated by the reaction of

TABLE I  
*Effect of Potassium Hydroxide on Activity of Synthetic Potato Polysaccharide*

Treatment	Inorganic P liberated in 10 min.
	mg.
Saturated solution of polysaccharide . . . . .	0.031
Dispersed in potassium hydroxide and neutralized . . . . .	0.130
Same after 48 hrs. . . . .	0.047
Blank (no polysaccharide added) . . . . .	0.015

the latter with iodine. Sumner *et al.* (8), in a preliminary note, reported that "the nature of the product synthesized from Cori ester by plant phosphorylase depends upon the kind and amount of carbohydrate added to prime the reaction."

Two sources of activator polysaccharides were used, the non-dialyzable dextrans from fat-free corn-starch which had been hydrolyzed to the achromic point with hydrochloric acid, and synthetic polysaccharide hydrolyzed in stages ranging from 5 to 750 minutes. The dextrans were prepared by neutralizing the hydrolyzed starch with sodium hydroxide, dialysis until free from chlorides, concentration *in vacuo* to a small volume, and then precipitation with methanol. The precipitate was dried and used in the concentrations given in Table II. To determine the activating ability of the dextrans and partially hydrolyzed synthetic polysaccharide, the same methods were used as described previously. At the end of the incubation periods, ranging from 5 to 40 minutes, the reactions were stopped by adding 0.2 ml. of 0.5 per cent iodine in 1.0 per cent potassium iodide. The absorption characteristics of the different samples were studied by means of a Coleman spectrophotometer.

As indicated in Table II the color formed by iodine and the carbohydrate synthesized during the first few minutes tended to be about the same as that given by iodine and the activator polysaccharide, even though the latter ranged from blue to colorless, depending upon the degree of acid hydrolysis it was subjected to. However, prolongation of the polysaccharide-synthesizing reaction results in products that stain blue with iodine irrespective of the character of the activator. One possible explanation seems to be that the synthesis is essentially the formation of amylose type chains in combination with the activator polysaccharide, somewhat as suggested by Cori *et al.* (7), and that, within limits, the length

TABLE II

*Degree of Acid Hydrolysis of Activating Polysaccharide and Iodine Color Reaction of Synthesized Products*

Activator		Activity (inor- ganic P liber- ated in 10 min.)	Iodine color reaction of					
			Activator	Synthesized products formed during in- cubation periods of				
				5 min.	10 min.	20 min.	40 min.	
Synthesized potato poly- saccharide hydrolyzed for	<i>min.</i>	<i>mg.</i>						
	20	0.037	Blue- green	Pink	Laven- der	Blue	Dark blue	
	510	0.405	Yellow	Red	Purple	“	Blue	
	750	0.098	“	Yellow	Dark yellow	Laven- der	Purple	
	<i>mg per 3.5 ml.</i>							
Dialyzed corn- starch dextrin	0.5	0.224	Yellow- brown	Laven- der	Laven- der	Blue	Dark blue	
	20.0	0.629	“	Yellow- brown	Brown	Brown- red	Purple	

of the chain is determined by the reaction time. Thus a short reaction time, especially with large amounts of activator, would yield amyloextrins giving a different color with iodine than products formed by prolonged enzymatic action.

## SUMMARY

A convenient method of concentrating phosphorylase from potatoes has been described. The factors of importance in determining phosphorylase activity have been studied and on the basis of the results slight modifications of the Green and Stumpf analytical method have been made.

The ability of different polysaccharides to activate potato phosphorylase increases progressively to the neighborhood of the achromic point, when hydrolyzed with acid, and then diminishes as hydrolysis is continued.

Amylose type polysaccharides, both natural and synthetic, exhibit this behavior as well as starches containing both branched and straight chain components. It appears probable that the phosphorylase activator need not contain more than 7 or 8 glucose units per molecule. Branched chain polysaccharides are unnecessary for the activation of potato phosphorylase.

Some attempt has been made to determine whether activator polysaccharides constitute patterns which regulate the nature (molecular weight, structural configuration, etc.) of the synthesized polysaccharides. The results are inconclusive.

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# A METHOD OF DETERMINING CARBONIC ANHYDRASE ACTIVITY BY THE USE OF UNIMOLECULAR VELOCITY CONSTANTS

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Recent work in this laboratory necessitated the measurement of carbonic anhydrase activity in the blood and reproductive system of hens.

The method commonly used is that of Meldrum and Roughton (1), who defined the unit  $E$  by the equation  $E = (R - R_0)/R_0$  where  $R_0$  is the reciprocal of the time taken for the second quarter of the carbon dioxide to be evolved when no catalyst is present and  $R$  is the reciprocal of the time taken for the second quarter of the evolution when the catalyst is present. Unit  $E$  is a linear function of the amount of catalytic material added when the rate of the process is not too rapid. Meldrum and Roughton stated, however, that  $E$  is not a linear function of the amount of catalytic material when large amounts are present.

It seems that the unimolecular velocity constants for the decomposition of carbonic acid might provide a simple method of measuring carbonic anhydrase activity. An effort was made, therefore, to devise another unit for measuring carbonic anhydrase activity.

The rate of decomposition of carbonic acid was measured by the manometric method of Meldrum and Roughton, except that the sodium bicarbonate solution used contained 0.1500 mole of sodium bicarbonate and 0.0222 mole of sodium hydroxide per liter of solution. Measurements were made at 15°.

Velocity constants were calculated by the method of Guggenheim (2), which has the advantage that each observation is used only once, and it is not necessary to follow the reaction to completion. Pressure readings  $x_1, x_2, \dots, x_n$  were taken at equal intervals of time (twelve readings at 15 second intervals in this case). The first reading was taken 30 to 45 seconds after mixing. The readings were paired for 90 second intervals and differences taken; *i.e.*,  $(x_7 - x_1), (x_8 - x_2), \dots, (x_{12} - x_6)$ . The velocity constant was obtained by plotting the common log of the differences against time. The slope of the line multiplied by 2.303 gives the velocity constant. A typical graph is shown in Fig. 1.

The amount of enzyme activity was found by measuring the velocity

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constants for the uncatalyzed and catalyzed reactions. The activity is expressed by the unit  $k_a$ , defined by the equation,  $k_a = (k_c - k_0)/C$ , where  $k_c$  equals the velocity constant of the catalyzed reaction,  $k_0$  equals the velocity constant of the uncatalyzed reaction,  $k_a$  equals the increase in the velocity constant per unit of catalyzed material, and  $C$  equals the

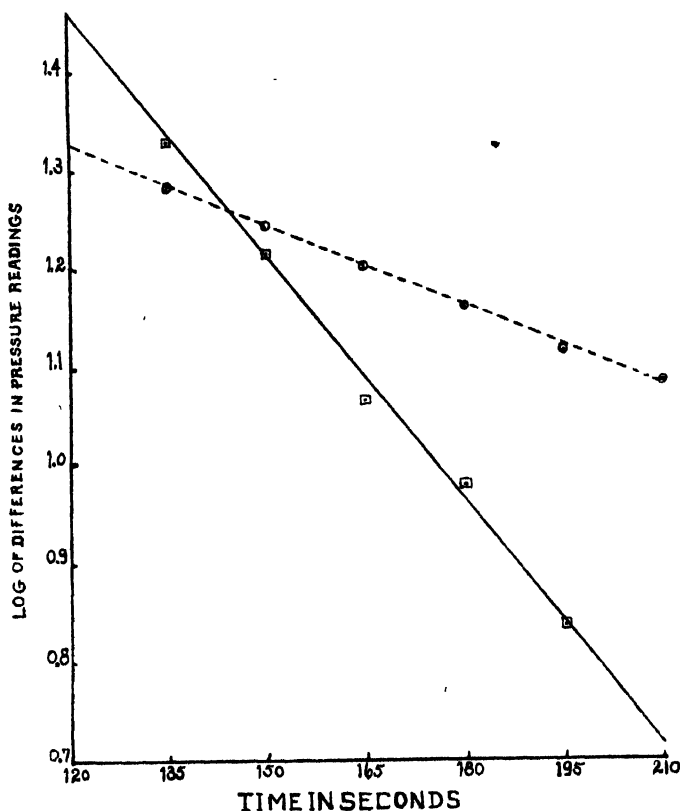


FIG. 1. Graphical method of calculating velocity constants. The slope of the line multiplied by 2.303 gives the value of  $k$ . The dotted line is for the uncatalyzed reaction; the solid line, for the catalyzed reaction.

concentration of the enzyme. This will be proportional to the volume of liquids or weight of solids containing the enzyme, and may be expressed in terms of ml. of liquids or gm. of solids.

The unit  $k_a$  is a linear function of the activity of the enzyme at the concentrations studied. This statement is illustrated in Fig. 2, in which the value of  $(k_c - k_0)$  is plotted against the ml. of blood used to supply the carbonic anhydrase.

When the carbonic anhydrase activity of a sample of blood was calculated by the two methods, from the same kinetic data, Meldrum and Roughton's method gave a value of  $E = 325$  units per ml. of blood, whereas this method gave a value of  $k_d = 2.08$  units per ml. of blood.

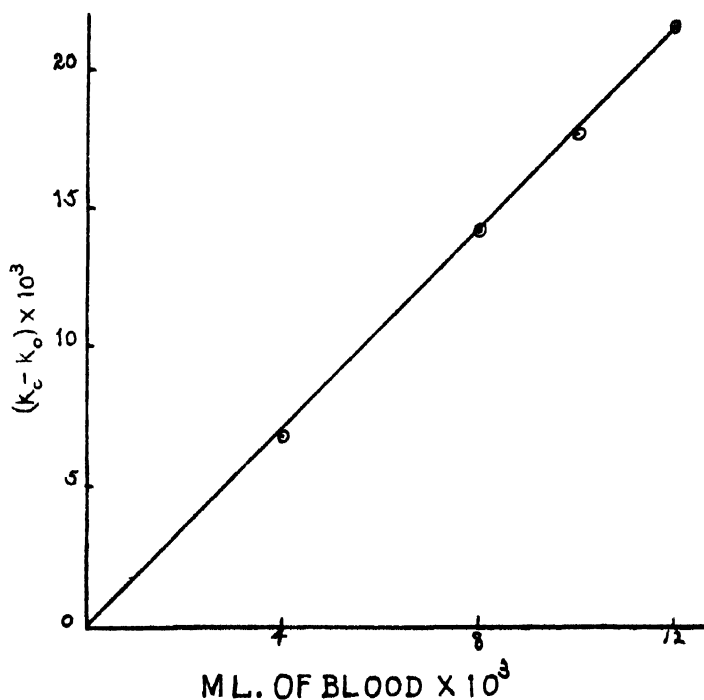


FIG. 2. The relation between the increase in the velocity constant ( $k_c - k_0$ ) and the concentration of the enzyme.

This method has given good results with two different observers. The value of  $k_d$  for hen blood varies from 2 to 5 units when the concentration is expressed in ml. of blood.

The authors are indebted to Dr. Marie S. Gutowska for her cooperation in this work. Thanks are also due to the Poultry Department of the Massachusetts State College for supplying the hens used in this investigation.

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# GROWTH INHIBITION BY ANALOGUES OF PANTOTHENIC ACID

## III. N-PANTOYLALKYLAMINES AND RELATED COMPOUNDS\*

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In Paper I of this series (2), it was shown that N-pantoylethylamine and N-pantoyl-*n*-propylamine inhibited growth of *Leuconostoc mesenteroides* P-60, and that this inhibition was not apparent in the presence of excess pantothenic acid. Since the *n*-propylamide was twice as effective as an inhibitor as the ethylamide, the preparation of additional alkylamides of the same type was undertaken. In addition, a number of other related compounds have been prepared. The preparation of these and their effect on several microorganisms which require pantothenic acid for growth are described below.

### EXPERIMENTAL

*dl*-N-Pantoylalkylamines--To 1.3 gm. of *dl*-pantolactone, an excess of 10 per cent over the calculated quantity of alkylamine was added. The reaction mixture became slightly warm in all cases and was then heated with occasional stirring at 100–110° for 2 hours at atmospheric pressure and for an additional 2 hours under reduced pressure to remove unchanged reactants. The yields of crude viscous products amounted to 85 to 95 per cent of the theoretical based on the lactone. On standing overnight only *dl*-N-pantoylisoamylamine crystallized and was recrystallized from ether containing a small amount of absolute alcohol. Each of the remaining products was dissolved in 5 cc. of ether and allowed to stand in a refrigerator overnight. *dl*-N-Pantoylisobutylamine and *dl*-N-pantoylisopropylamine, which crystallized by this procedure, were recrystallized from ether. Small amounts of the viscous oily material from the preparation of pantoyl derivatives of *sec*-butylamine, *n*-butylamine, and *n*-amylamine covered with petroleum ether crystallized after standing for several days in a refrigerator. Upon addition of petroleum ether to ether solutions of the compounds and seeding with the crystals obtained in this way, the three compounds crystallized and were recrystallized from ether-petroleum ether. The derivative from *n*-heptylamine could not be induced

\* For Paper II of this series see Shive and Snell (1).

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to crystallize by any of these methods and was subsequently distilled under a pressure of  $10^{-6}$  mm. with an outside bath temperature of 123–128°. All of the other derivatives crystallized as colorless prisms, melting as listed in Table I. Two isomers would be expected from *sec*-butylamine; so the mother liquor from the first crystallization was evaporated to obtain a viscous liquid which was dissolved in petroleum ether containing a small amount of ether. On cooling and seeding, more of the least soluble product was obtained. The mother liquor was again evaporated to obtain a viscous oil which was heated under reduced pressure to remove the solvent. This residual material was tested together with the crystalline material to determine whether there was any great difference in their inhibitory properties.

*dl*-*N*-Pantoyl- $\beta$ -Methoxyethylamine— $\beta$ -Methoxyethylamine was condensed with *dl*-pantolactone as described above. After all attempts to

TABLE I  
*dl*-*N*-Pantoylalkylamines

Compound	M.p.	Formula	N calculated	N found
			per cent	per cent
<i>dl</i> - <i>N</i> -Pantoylisopropylamine.....	50.5–52.5	$C_9H_{19}O_2N$	7.40	7.53
<i>dl</i> - <i>N</i> -Pantoyl- <i>n</i> -butylamine.....	51.6–53.4	$C_{10}H_{21}O_2N$	6.89	7.01
<i>dl</i> - <i>N</i> -Pantoylisobutylamine.....	65.0–67.0	$C_{10}H_{21}O_2N$	6.89	7.06
<i>dl</i> - <i>N</i> -Pantoyl- <i>sec</i> -butylamine.....	53.0–56.0	$C_{10}H_{21}O_2N$	6.89	7.10
<i>dl</i> - <i>N</i> -Pantoyl- <i>n</i> -amylamine.....	43.5–45.0	$C_{11}H_{23}O_2N$	6.45	6.32
<i>dl</i> - <i>N</i> -Pantoylisoamylamine.....	76.0–78.0	$C_{11}H_{23}O_2N$	6.45	6.58
<i>dl</i> - <i>N</i> -Pantoyl- <i>n</i> -heptylamine.....	Liquid	$C_{13}H_{27}O_2N$	5.71	5.76

obtain a crystalline product failed, the product was distilled under a pressure of  $10^{-6}$  mm. at an outside bath temperature of 120–125°.

*Analysis*— $C_9H_{19}O_2N$ . Calculated, N 6.82; found, N 6.90

*dl*-*N*-Pantoyl- $\beta$ -phenylethylamine—The condensation of *dl*-pantolactone with  $\beta$ -phenylethylamine was carried out as described above. The viscous liquid product was dissolved in ether and on cooling crystallized readily. After recrystallizing from ether containing a small amount of absolute alcohol, the colorless prisms melted at 89.6–91.0°.

*Analysis*— $C_{14}H_{21}O_2N$ . Calculated, N 5.57; found, N 5.77

*dl*-Pantothenonitrile— $\beta$ -Aminopropionitrile was prepared from ammonium hydroxide and acrylonitrile by the method of Buc, Ford, and Wise (3). A solution of 5.2 gm. of *dl*-pantolactone and 2.8 gm. of  $\beta$ -aminopropionitrile in 15 cc. of ether was refluxed for 2 hours. The ether was re-

moved from the oily layer by distillation and was replaced by petroleum ether. On cooling, the oily layer covered with petroleum ether gradually crystallized. The crystals, filtered and washed with cold petroleum ether, weighed 7.6 gm. and constituted a yield of 95 per cent of the theoretical. Recrystallization from chloroform gave colorless prisms, m.p. 67.0–68.5°.

*dl-Pantothenylamine*—Hydrogenation of 4.5 gm. of pantothenonitrile in 30 cc. of absolute ethanol was effected with Raney's nickel catalyst in a

*Analysis*— $C_8H_{15}O_2N_2$ . Calculated, N 13.99; found, N 13.75

TABLE II

*Susceptibility of Leuconostoc mesenteroides P-60 to Inhibition by Pantothenic Acid Analogues*

Incubated 20 hours at 30°.

Inhibitor	Molar ratios (analogue to pantothenic acid) for maximum inhibition
<i>dl</i> -Pantothenyl alcohol.....	350
<i>dl</i> -N-Pantoylethylamine.....	10,000*
<i>dl</i> -N-Pantoyl- <i>n</i> -propylamine.....	5,000*
<i>dl</i> -N-Pantoyl- <i>n</i> -butylamine.....	750
<i>dl</i> -N-Pantoyl- <i>n</i> -amylamine.....	1,500
<i>dl</i> -N-Pantoyl- <i>n</i> -heptylamine†.....	2,000
<i>dl</i> -N-Pantoylisopropylamine.....	25,000
<i>dl</i> -N-Pantoylisobutylamine.....	2,500
<i>dl</i> -N-Pantoylisoamylamine.....	5,000
<i>dl</i> -N-Pantoyl- <i>sec</i> -butylamine (m.p. 53–56°) ..	75,000
<i>dl</i> -N-Pantoyl- <i>sec</i> -butylamine‡.....	75,000
<i>dl</i> -N-Pantoyl- $\beta$ -methoxyethylamine.....	4,000
<i>dl</i> -N-Pantoyl- $\beta$ -phenylethylamine.....	15,000
<i>dl</i> -Pantothenonitrile§.....	10,000
<i>dl</i> -Pantothenylamine§.....	40,000

\* Data from previous work (2).

† Irreversibly toxic at levels greater than 10 mg. per 10 cc.

‡ Sample obtained from solvent in crystallizing the stereoisomeric form, m.p. 53–56°.

§ Sterilized by filtration and added aseptically to sterilized medium.

glass-lined bomb at a pressure of 2000 pounds per sq. in. and at a temperature of 80°. After filtering the catalyst from the solution and removing the ethanol by first distilling under atmospheric pressure and finally under reduced pressure, a product was obtained which contained 4.04 per cent amino nitrogen (Van Slyke). This represents a purity or yield of 59 per cent. All attempts to obtain a crystalline product failed; the product was therefore molecularly distilled under a pressure of  $10^{-8}$  mm. with a bath temperature of 115–120°. Higher temperatures must be avoided, since

they cause decomposition of the amine, as evidenced by an increase in pressure.

*Analysis*— $C_9H_{18}O_2N(NH_2)$ . Calculated, amino N 6.86; found, 7.24

*Testing Methods*—The methods of biological testing have been previously described (2). Temperature of incubation for *Lactobacillus ara-*

TABLE III  
Comparative Susceptibility of Various Organisms to Inhibition by Pantothenic Acid Analogues

Inhibitor	Molar ratios (analogue to pantothenic acid) for indicated inhibition of organism					
	<i>Leuconostoc mesenteroides</i> P-60		<i>Lactobacillus arabinosus</i> 17-5		<i>Lactobacillus casei</i>	
	Half maximum	Maximum	Half maximum	Maximum	Half maximum	Maximum
<i>dl</i> -Pantothenyl alcohol . . . . .	175	350	1,500	10,000	5,000	20,000
<i>dl</i> -N-Pantoyl- <i>n</i> -propylamine . . . . .	1,500*	5,000*	1,000	7,500	3,000	10,000
<i>dl</i> -N-Pantoyl- <i>n</i> -butylamine . . . . .	350	750	1,250	7,500	3,250	15,000
<i>dl</i> -N-Pantoyl- <i>n</i> -amylamine . . . . .	600	1,500	1,500	10,000	1,250	6,000
<i>dl</i> -N-Pantoyl- <i>n</i> -heptylamine† . . . . .	450	2,000	750	4,500	1,000	4,250
<i>dl</i> -N-Pantoylisoamylamine . . . . .	2,500	5,000	3,000	20,000	10,000	50,000
<i>dl</i> -N-Pantoyl- $\beta$ -phenylethylamine . . . . .	5,000	15,000	12,000	40,000	4,000	10,000

\* Data from previous work (2).

† Irreversibly toxic at levels greater than 10 mg. per 10 cc. of media.

TABLE IV  
Effect of Incubation Time on Inhibition of *Lactobacillus casei* by Pantothenic Acid Analogues

Incubation time	Inhibition	Molar ratios (analogue to pantothenic acid) for indicated inhibition	
		<i>dl</i> -Pantothenyl alcohol	<i>dl</i> -N-Pantoyl- <i>n</i> -amylamine
<i>hrs.</i>			
17	Half maximum	2,500	750
	Maximum	15,000	5,000
28	Half maximum	5,000	2,500
	Maximum	33,000	12,500
66	Half maximum	5,000	2,500
	Maximum	90,000	50,000

*binosus* 17-5 and *Leuconostoc mesenteroides* P-60 was 30°, for *Lactobacillus casei* 37°. The time of incubation was 20 hours unless otherwise noted. The data in Tables II, III, and IV were obtained by adding 0.2  $\gamma$  of calcium pantothenate per 10 cc. of medium and varying the amount of inhibitor to determine the molar ratios at which growth was inhibited.

**Results**—From previous work (2), growth of *Leuconostoc mesenteroides* was known to be sensitive to inhibition by several N-pantoylalkylamines similar to those described above. This organism was used as the initial test organism for all the new compounds. Results of these tests are listed in Table II. The results are expressed as "molar inhibition ratios"; *i.e.*, the ratio of the molar concentration of the inhibitor to that of calcium pantothenate at which maximum inhibition of growth resulted. Under a standard set of conditions, this ratio is constant over a rather wide range of concentration of pantothenate. This is usually characteristic of competitive inhibition. Of the analogues listed in Table II, six were studied further with *Lactobacillus arabinosus* and *Lactobacillus casei* as the test organisms. The results of these tests are given in Table III. At levels above 10 mg. per 10 cc., growth inhibition by *dl*-N-pantoyl-*n*-heptylamine was not prevented by increasing the concentration of calcium pantothenate. This was the only instance in which an irreversible toxicity was observed. Inhibition by this substance, too, was competitive at concentration levels below this limiting value. The effect of increasing the time of incubation on the molar inhibition ratios obtained is shown in Table IV.

#### DISCUSSION

Inhibition of growth of several organisms which require pantothenic acid is produced by each of the compounds described above. This inhibition is similar to that previously described for other analogues of pantothenic acid (1, 2), since it is counteracted specifically by pantothenic acid, and becomes apparent only when the *ratio* of analogue to pantothenic acid surpasses a critical value. This competitive relationship exists over a wide range of concentrations. Only with N-pantoyl-*n*-heptylamine was irreversible toxicity observed, and this only at high levels.

The susceptibility of any given organism to inhibition by the various compounds varies markedly with structure, even with such similar compounds as the N-pantoylalkylamines. As previously noted with other analogues (1, 2), the comparative potencies of members of a series of inhibitors for one organism cannot be predicted from a knowledge of their comparative potencies for another organism. For *Leuconostoc mesenteroides*, none of the alkylamides was as effective as pantothenyl alcohol. Their effectiveness increased considerably as the length of the alkyl group was increased to 4 carbon atoms, then slowly decreased. In all cases, a compound containing a branched alkyl group was less active than its isomer containing the *n*-alkyl group.

For *Lactobacillus arabinosus* and *Lactobacillus casei*, several of the alkylamides were more effective inhibitors than pantothenyl alcohol; neither organism is as sensitive to any of these analogues as to some others which



are available (1, 2). No definite trend in effectiveness with increasing chain length is apparent with these organisms, for both the *n*-propyl- and the *n*-heptylamides are more effective than the intervening members of the series. The effectiveness of the *n*-heptylamide may be connected with its low solubility in water. At high concentrations (1 mg. per cc. of medium) it alters the surface tension of the medium rather markedly; its irreversible toxicity at these higher levels may be connected with this behavior.

In general, pantothenonitrile, pantothenylamine, and *N*-pantoyl- $\beta$ -phenylethylamine were rather poor inhibitors for the organisms tested; thus substitution of negative groups for the carboxyl group of pantothenic acid does not seem to produce especially effective inhibitors. Unless special precautions are taken, pantothenonitrile hydrolyzes to yield sufficient pantothenic acid to mask its inhibitory properties. *N*-Pantoyl- $\beta$ -methoxyethylamine was only slightly more effective than *N*-pantoyl-*n*-propylamine.

From the data of Table IV, it is evident that the amount of analogue required to inhibit growth increases with the time of incubation. This is especially true if one determines the amount necessary just to prevent visible growth; the amount which permits half maximum growth is much less variable. Similarly, values for the ratios listed vary slightly with the amount of inoculum used, its age, and other factors. Under constant conditions, however, the values are rather reproducible.

Micro-Dumas and Van Slyke analyses were carried out by Gwyn White Shive.

#### SUMMARY

Preparation and physiological properties of a series of *dl*-*N*-pantoyl-alkylamines, and of *dl*-*N*-pantoyl- $\beta$ -methoxyethylamine, *dl*-*N*-pantoyl- $\beta$ -phenylethylamine, *dl*-pantothenonitrile, and *dl*-pantothenylamine are reported. All of these compounds inhibit growth of various organisms which require pantothenic acid; the inhibition is competitive in nature, and is specifically alleviated by excess pantothenic acid. The compounds vary markedly in inhibitory properties for a single organism; similarly, the relative potencies of members of a series of compounds differ markedly for different organisms. These results confirm and extend those obtained previously with other inhibitory analogues of pantothenic acid (1, 2, 4).

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# THE PREPARATION AND BIOLOGICAL ACTIVITY OF SOME RIBOFLAVIN DERIVATIVES

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The quest for soluble derivatives of riboflavin has resulted in the description of several compounds of this nature. Relatively few of these have been well characterized, however, from the standpoint of biological or microbiological activity, due, in part, to the circumstance that some of these preparations were described before the advent of refined assay methods. Snell and Strong (1) reported careful microbioassays of a number of synthetic flavins, which had been prepared and bioassayed in various laboratories. They concluded that "insofar as the selection of active or inactive compounds is made the basis for comparison, the correlation between the rat assay and the present bacterial test seems rather close. A comparison of the degree of activity is at present rendered impossible by the paucity of data concerning the relative potency of the various active flavins on rats." We have found no later efforts which attempt to relate these types of assay to flavin structure. The present report concerns such comparative measurements on a number of soluble riboflavin derivatives.

The riboflavin molecule offers two general possibilities for ready substitution, *viz.* in the ring at the imino nitrogen or in the ribose chain. The former possibility was explored by Kuhn and Rudy (2), who found that such compounds were biologically inactive and did not fluoresce. Substitution in the ribose chain, however, can result in biologically active derivatives. Those which have been described can be classified into two main groups, esters and acetals. Included among the esters which have been described are the phosphate (3-5), acetates (4, 6-8), borate (9), and tetrabenzoate (9). The phosphate and acetates were shown to be biologically active, but no tests for activity are recorded for the boric acid and tetrabenzoic acid esters. The present report presents data on the preparation and activity of succinic acid esters. Included among the acetals are the mono- and diacetone derivatives described by Kuhn *et al.* (10), who claimed that the diacetone compound is active (6). It will be shown in the present report that two other compounds of the acetal type, obtained by condensation of chloral and of levulinic acid with riboflavin, have no vitamin activity.

*Preparation of Compounds*—A detailed account of the methods of prep-

aration will be found in the experimental section. Essentially, the succinic acid esters were prepared by condensation of riboflavin with succinic anhydride in pyridine. The extent of substitution was controlled by varying the molecular ratio of succinic anhydride to riboflavin from 1 to 4.<sup>1</sup>

The acetals were made according to a procedure of Coles, Goodhue, and Hixon (11) for the preparation of chloraloses, in which a polyhydroxy compound (riboflavin) is treated in sulfuric acid solution with excess aldehyde or ketone (chloral, levulinic acid).

*Assay Methods*—All compounds were assayed by fluorometric (12), microbiological (13), and biological (rat)(14) procedures. For bioassay, Labco rice polish was substituted for the rice bran concentrate used by Street (14).

TABLE I

*Effect on Microbioassays of Autoclaving Riboflavin Succinates with Medium Versus Filtration through Glass Bacterial Filters*

All values are given as per cent of riboflavin in the compound.

Treatment	Method of measurement	Succinate			
		Mono-	Di-	Tri-	Tetra-
Autoclaved 20 min. at 15 lbs.	Turbidimetric	62	33	11	5.3
	Titrimetric	63	33	12	5.5
Filtered through glass	Turbidimetric	43	13	0.0	0.0
	Titrimetric	46	14	0.0	0.0

In order to minimize hydrolysis of the compounds studied, the following precautions were observed.

The solutions of the compounds were prepared at room temperature.

The final solutions used for microbioassay were prepared aseptically by filtration through fritted glass bacterial filters.<sup>2</sup> Aliquots of these filtrates were then added by sterile pipette to the autoclaved medium. Comparative trials by this and the orthodox autoclave method (Table I) showed distinctly higher values by the latter, which can be ascribed only to hydrolysis.

The microbioassays were read both after 1 day at 37° (turbidimetric) and after 3 days (titrimetric) in order to ascertain whether the longer

<sup>1</sup> This method of preparation provides no guarantee of the homogeneity of the di- and trisuccinates, but rather a predominance of one particular homologue. The presence of small amounts of other homologues does not vitally affect the validity of the vitamin assays by the various methods, but may exert considerable influence on the solubility measurements. Solubility data are presented nevertheless because replicate preparations of the compounds gave results of the same order of magnitude.

<sup>2</sup> Seitz filters were tried first. It was found that considerable adsorption of the various flavins occurred on the Seitz filter pads. Filtration of large volumes of the solutions provided no relief, since the pads displayed an extensive capacity for adsorption.

incubation at 37° caused any hydrolysis. As shown in Table I, no appreciable effect was found for the succinate derivatives. These data also show that the rate of utilization of the active compounds by *Lactobacillus casei* was the same as for riboflavin, even though the extent of utilization did not reach theoretical values for any of them.

**Activity of Riboflavin Succinates**—Fig. 1 shows the levels of response obtained with the three assay methods in relation to the extent of substitution with succinic acid. The fluorometric values are in good agreement with the theoretical in all cases, and hence can serve as a measure of the concentration of these compounds. Bioassay showed the mono-

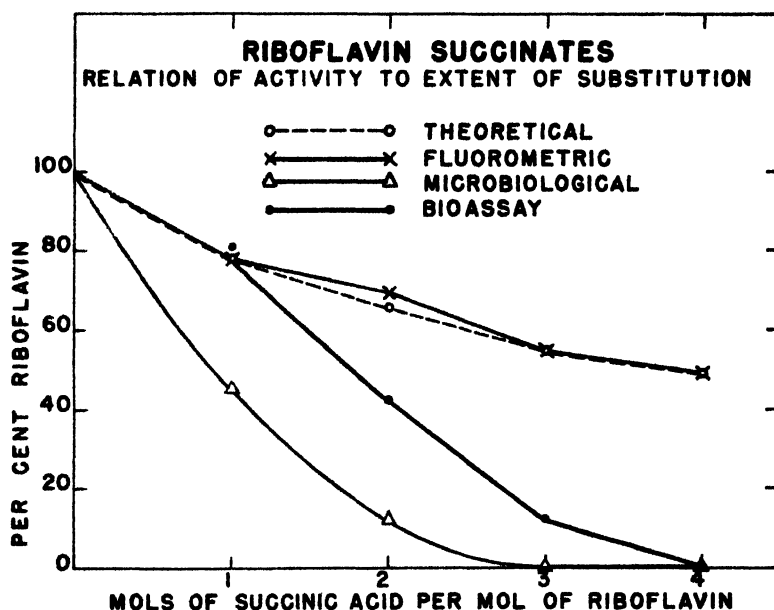


FIG. 1

succinate to be fully active,<sup>3</sup> the disuccinate partially active, the trisuccinate slightly active, and the tetrasuccinate completely inactive. The microbiological values ran parallel to but distinctly lower than the biological.

The biological inactivity of the tetrasuccinate, which we observed in both curative and prophylactic trials, may be contrasted with the reported

<sup>3</sup> The monosuccinate was found to be fully active both when administered by mouth and by intramuscular injection. The di- and trisuccinates were tested by feeding only, while the tetrasuccinate was found to be inactive both by mouth and by injection.

activity of the tetraacetate (15). The inactivity of the latter for lactic acid bacteria (1) suggests that its activity in the rat is due to hydrolysis in the animal. It would appear, therefore, that the tetrasuccinate is not hydrolyzed by the rat to the more active lower homologues or to riboflavin.

It is more difficult to interpret the low microbiological results, particularly with regard to the monosuccinate, which is completely active by bioassay. In an attempt to explain these data, the following aspects were studied on the monosuccinate but found *not* to influence the low values. (1) The age of the culture. Rapidly growing, young cultures, obtained by serial daily transfer for 5 days, gave the same response as an older culture transferred from stock 24 hours before use, the stock culture being transferred monthly. (2) The presence in the riboflavin standard of succinic acid in amounts equal to or twice as great as the amount which would be derived from the monosuccinate on complete hydrolysis. (3) Enzyme treatment with 2 per cent of clarase (16). However, complete hydrolysis leading to theoretical riboflavin values was achieved by heating in  $N$  HCl at  $100^{\circ}$  for 30 minutes. Complete hydrolysis was also obtained by heating in  $0.1\ N$  HCl at 15 pounds pressure ( $122^{\circ}$ ) for 30 minutes, which is the extraction procedure of the United States Pharmacopoeia (17).

Since we have been unable to elicit any analytical cause for the low microbiological responses, we must conclude for the present that *these succinate derivatives cannot be utilized as fully by Lactobacillus casei* as by the rat. There is some precedent for this finding in the work of Snell and Strong (1), who found that four of a group of thirteen flavins examined supported growth of *Lactobacillus casei* or *Streptococcus faecalis* as the sole source of flavin. Of these four, only one, however, 6-ethyl-7-methyl-9-(*d*,1'-ribityl)-isoalloxazine, approached the activity of riboflavin, while the other three showed lower quantitative responses. From the standpoint of bioassay, however, all four were classified as "active on rats." The similarity ends there, since none of the four had substituents in the ribose chain.

*Solubility of Riboflavin Succinates*—Solubility was determined by mechanical shaking with excess solute at  $25^{\circ}$  for 10 hours, filtering, and determining the concentration of solute in the filtrate by the fluorometric method; as previously noted, Fig. 1 shows that the fluorometric assay is adequate for this purpose. Fig. 2 demonstrates the marked increase in solubility in water that occurs as the number of succinate substituents increases. Since these compounds have free carboxyl groups, salts may be prepared. It was found that the sodium, monoethanolamine, and diethanolamine salts were even more soluble than the corresponding esters. Since riboflavin monosuccinate is the only member of the series which is fully active by bioassay, the solubility of this compound and its sodium

salt were studied more extensively. Representative data are shown in Table II.

*Acetals*—The condensation products of riboflavin with chloral and with levulinic acid were fluorescent but completely inactive by both microbiological and biological assays. The inactivity of these acetals may be con-

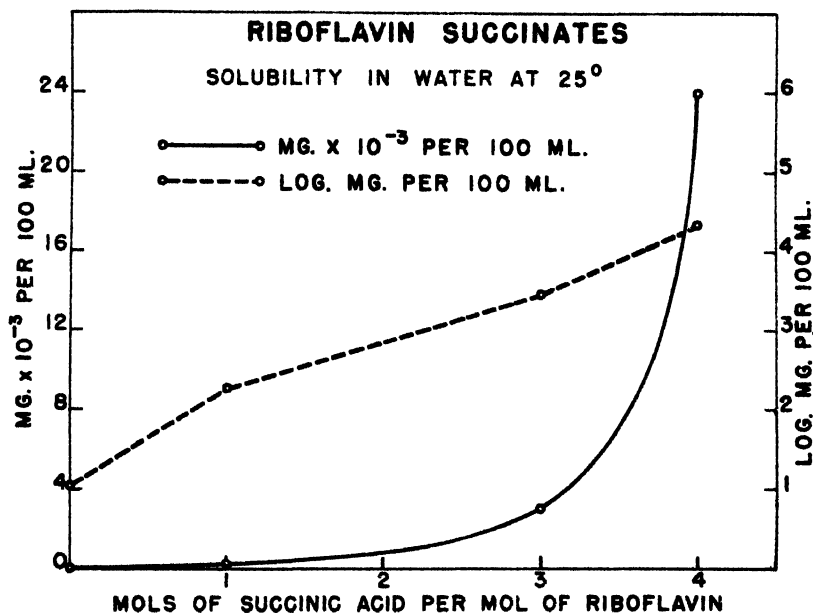


FIG. 2

TABLE II

*Solubility of Riboflavin Monosuccinate in Various Solvents at 25°*

All values are expressed in terms of riboflavin, mg. per 100 cc.

Solvent	Riboflavin	Riboflavin monosuccinate	Sodium salt of riboflavin monosuccinate
Water. . . . .	11	105	250
Ethanol (95%). . . . .	4.5	30	24
Glycerol . . . . .	25	176	350
Propylene glycol. . . . .	22	250	580

trasted with the claim (6) that diacetone riboflavin is biologically active, the latter being the only acetal for which activity has been reported, We were unable to find the data upon which Kuhn (6) based the claim for diacetone riboflavin, and hence must reserve judgment on relationships among acetals as a group.

## EXPERIMENTAL

*Riboflavin Monosuccinate*—1 gm. of riboflavin was refluxed with 300 mg. of succinic anhydride (1.1 moles) in 200 cc. of dry pyridine. The mixture became homogeneous after 2 to 2.5 hours; heating was continued for 1 hour thereafter. The pyridine was then distilled off *in vacuo*; the residue was completely freed of pyridine by drying *in vacuo* at 100°, and then recrystallized from water. 800 mg. of a yellow substance were obtained, melting at 245°, corrected, with decomposition.

*Analysis*— $C_{21}H_{24}O_8N_4$

Calculated. C 52.91, H 5.05, riboflavin 79.0

Found. " 52.55, " 5.19, " (fluorometric) 78

*Riboflavin Disuccinate*—2 gm. of riboflavin were refluxed with 1.2 gm. of succinic anhydride (2.2 moles) in 80 cc. of dry pyridine for 3 hours. The pyridine was distilled off *in vacuo* and the residue recrystallized from water. 2 gm. of yellow substance resulted, m.p. 223°, corrected, with decomposition.

*Analysis*— $C_{23}H_{26}O_{12}N_4$ .

Calculated. C 52.06, H 4.90, N 9.72, riboflavin 65.2

Found. " 51.72, " 5.50, " 10.00, (fluorometric) 68

*Riboflavin Trisuccinate*—2 gm. of riboflavin were refluxed with 1.8 gm. (3.3 moles) of succinic anhydride in 30 cc. of dry pyridine for 3 hours. The pyridine was distilled off *in vacuo*, and the residue dissolved in absolute ethanol and precipitated with ether. 2.4 gm. of a yellow solid were obtained, which were redissolved in absolute ethanol and reprecipitated with ether. The substance melted at 120–132°.

*Analysis*— $C_{25}H_{32}O_{15}N_4$ .

Calculated. Riboflavin 55.6

Found (fluorometric) 56

*Riboflavin Tetrasuccinate*—2 gm. of riboflavin were refluxed with 2.4 gm. (4.4 moles) of succinic anhydride in 20 cc. of dry pyridine for 2 hours. The pyridine was distilled off *in vacuo* and the residue taken up with absolute alcohol and precipitated with ether. 3.2 gm. of a light yellow solid were obtained and recrystallized from acetone; m.p. 112–115°, corrected.

*Analysis*— $C_{27}H_{36}O_{18}N_4$

Calculated. C 50.91, H 4.68, N 7.22, riboflavin 48.5

Found. " 50.28, " 4.76, " 7.37, " (fluorometric) 48

*4',5'-Trichloroethylidene Riboflavin*—The method of Coles *et al.* (10) for the preparation of chloraloses was followed. 17 gm. of chloral hydrate were stirred mechanically with 30 cc. of concentrated sulfuric acid, while 10 gm. of riboflavin were added in small portions, the whole being

kept at 17°. Stirring was continued for 6 hours, and the reaction mixture was left in the refrigerator overnight. It was then poured into 250 cc. of ice water and again left in the refrigerator overnight. The precipitate was then filtered off, with a yield of 13.6 gm. of a yellow solid, which melted at 68–90°, was soluble in chloroform and acetone, and very soluble in methanol and ethanol. When boiled in ethanol or methanol, precipitation occurred, due probably to the conversion of the dichloral to the monochloral derivative. The precipitate decomposed above 265°. It was moderately soluble in dioxane, slightly soluble in ethanol and methanol, insoluble in water.

• *Analysis*— $C_{19}H_{19}O_6N_4Cl$

Calculated. C 44.92, H 3.78, Cl 20.96, riboflavin 74.1

Found. " 46.52, " 3.90, " 20.11, " (fluorometric) 76  
46.41 4.20

The analytical data show that the product consists mainly of the monochloral derivative. The high carbon, hydrogen, and fluorometric values and the slightly low chlorine figure suggest small admixture of riboflavin, which may be due to further hydrolysis of the monochloral compound.

*Condensation of Riboflavin with Levulinic Acid*—10 gm. of riboflavin were stirred for 3 hours with 6.7 gm. of levulinic acid in 40 cc. of concentrated sulfuric acid. The solution was then poured into 1.5 liters of water and the sulfuric acid precipitated with excess barium carbonate. After standing overnight, the mixture was warmed to 70° and the precipitate filtered off. The filtrate containing the barium salt of the condensation product of levulinic acid with riboflavin, and some unchanged riboflavin was evaporated to dryness *in vacuo*. The residue was dissolved in hot water, whence, upon cooling, 2.8 gm. of a mixture of unchanged riboflavin and some barium levulinic riboflavin crystallized out and were filtered off. The filtrate was acidified with excess concentrated sulfuric acid and the precipitated barium sulfate filtered off. The filtrate was then adjusted to pH 2.7 with sodium carbonate, evaporated to dryness *in vacuo*, and the residue was extracted with hot methanol. On cooling, 3 gm. of an extremely water-soluble substance crystallized. As it still contained traces of inorganic salts, it was again recrystallized from methanol.

	N	Riboflavin
Calculated for 4',5'-(carboxymethylisopropylidene)riboflavin, $C_{22}H_{25}O_5N_4$ .....	11.81	79.8
Calculated for 2',3':4'5'-di(carboxymethylisopropylidene)riboflavin, $C_{27}H_{31}O_5N_4$ .....	9.79	65.7
Found .....	10.53	73



It is evident from these figures that the preparation represents a mixture of the mono and di compounds. Since the preparation was biologically inactive, no attempts were made to separate the components.

We are indebted to Dr. A. Steyermark and his staff for the microanalyses, and to Mr. E. De Ritter, Mr. L. Drekter, and Dr. R. L. Schuman for their cooperation in carrying out the other analyses.

#### SUMMARY

The preparation of two acetal derivatives of riboflavin and of several riboflavin succinates is described. All exhibit theoretical fluorescence values. Both the biological and microbiological activities of the succinates decrease in inverse relation to the extent of substitution, but the two sets of values are not equal, the microbiological being considerably lower. These results demonstrate that neither fluorescence nor microbiological response of *Lactobacillus casei* is necessarily a quantitative measure of vitamin activity in the mammal for such flavins.

The succinates exhibit remarkable increases in solubility with increasing substitution.

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# STUDIES ON THE KINETICS OF ENZYMES LIBERATING STREPTOCOCCUS FAECALIS-STIMULATING FACTOR\*

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Recent publications by Mims, Totter, and Day (2), Bird and coworkers (3), and Laskowski, Mims, and Day (4) have described preparations of enzymes which convert a microbiologically inactive precursor to substances which stimulate the growth of *Streptococcus faecalis* (*S. lactis* R) and *Lactobacillus casei*. The studies reported here were designed to determine the Michaelis dissociation constants (5) of the enzyme-substrate complexes with enzymes prepared from different sources, and to throw light, if possible, on the biological rôle of the transformations brought about by the action of these enzymes. The purifications of enzymes and substrate were only carried sufficiently far to insure the absence of undesirable blank values in the microbiological determinations. It is therefore not to be expected that the values of the dissociation constants obtained in these experiments will necessarily be similar to values obtainable when the enzymes and substrates are available in pure form. However, the values obtained with the use of crude substrate and enzymes are probably more applicable to the enzymes as they occur in their natural environment.

## EXPERIMENTAL

*Preparation of Enzymes*.—Preliminary experiments indicated that enzyme solutions from rat liver prepared as described by Mims, Totter, and Day (2) were not sufficiently free from inert protein for the purposes of this experiment. Rat liver enzyme precipitated by ammonium sulfate was therefore subjected to further purification. The ammonium sulfate precipitate obtained from 74 gm. of rat liver was dissolved in 70 cc. of 0.05 M acetate buffer, pH 5, and to it were added 3.5 cc. of a 0.5 saturated solution of benzoic acid in acetone. The precipitated benzoic acid removed

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the bulk of the inactive material as well as a moderate amount of the enzyme. The supernatant was cooled to 0°, and 2 volumes of cold 95 per cent alcohol were added. After a few minutes the precipitate was centrifuged off and immediately taken up in 26 cc. of 0.05 M phosphate buffer, pH 7. This solution was used for the experiments described below. It contained 4.2 units (4) and 1.6 mg. of protein per cc. This preparation maintained its activity for more than a month preserved under benzene in the refrigerator.

The chicken pancreas enzyme was prepared by the method of Laskowski, Mims, and Day (4). It contained 500 units per mg. of protein.

The potato enzyme was prepared by freezing ground unpeeled raw potatoes and expressing the juice of the thawed material. The juice was cooled to 0–5° and treated with 1.1 volumes of chilled 95 per cent alcohol. The precipitate was discarded. The supernatant was then treated while still cold with 2 additional volumes of cold alcohol. The precipitate was extracted with 0.05 M phosphate buffer, pH 7, and immediately centrifuged and the supernatant discarded. The residue was again extracted for 1 hour with more buffer, and the material centrifuged. The supernatant contained 2.3 units and 0.77 mg. of protein per cc. The yield of enzyme was only about 9 per cent. No satisfactory method for obtaining high yields of purified enzyme from potatoes was discovered.

The substrate for the enzyme reactions was a concentrate prepared from yeast extract (Difco) by a procedure similar to that described by Mallory *et al.* (6). It contained 5.58 mg. of total solids and 30  $\gamma$  (potency 40,000 (7)) of potential *Streptococcus faecalis*-stimulating factor per cc. The preformed factor content was only 0.1  $\gamma$  per cc. The preparation of a concentrate was necessary because of the relatively large buffering capacity of the original yeast extract. Each of the enzyme preparations was found to be capable of increasing microbiological activity of this concentrate to the same degree under appropriate conditions of substrate concentration, pH, and temperature.

Duplicate tubes containing 0.15, 0.3, 0.9, 1.5, 3, 6, 12, 20, and 30  $\gamma$  of potential *Streptococcus faecalis*-stimulating factor in 1 cc. were set up. To each tube was added 1 cc. of a solution containing about 0.4 unit of the enzyme to be tested, in 0.05 M phosphate buffer, pH 7, and the tubes were incubated at 32° for 30 minutes. Three additional sets of tubes were set up simultaneously for incubation 60, 120, and 240 minutes respectively.

After the period of incubation the tubes were removed and placed in a boiling water bath to stop enzymic action. Appropriate dilutions were made and suitable aliquots assayed for *Streptococcus faecalis*-stimulating factor by the folic acid method of Mitchell and Snell (7). From the values obtained the initial velocities of the reactions catalyzed by each enzyme were calculated.

## DISCUSSION

At low substrate concentrations the three enzymic reactions appeared to be of the first order and the initial velocities of the reactions were calculated from the first order reaction velocity constants. At higher substrate concentrations the velocities of the reactions dropped rapidly, indicating

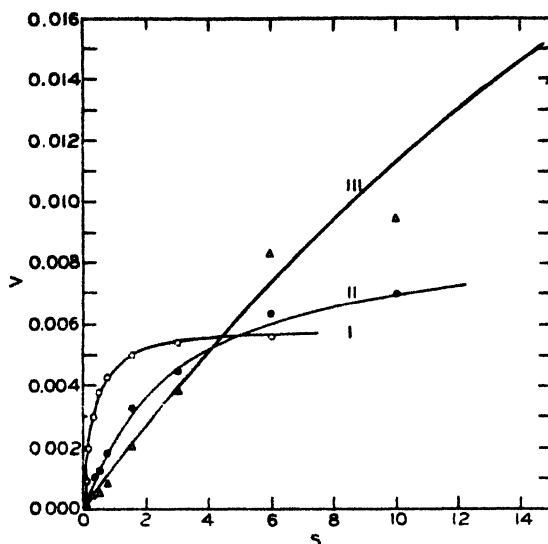


FIG. 1. Initial rates of enzymic liberation of *Streptococcus faecalis*-stimulating factor with increasing substrate concentrations at 32° and pH 7.  $V$  = mg. of material of potency 40,000 liberated per minute (ordinate);  $S$  = concentration of substrate in units, equivalent to 1 mg. of material of potency 40,000, per liter. Curve I, rat liver enzyme; Curve II, chicken pancreas enzyme; Curve III, potato enzyme.

TABLE I  
Summary of Michaelis Constants and Maximum Velocities

Source of enzyme	$K_s$ mg. per liter*	$V_{max}$ mg. per liter per min.*
Rat liver....	0.29	0.0059
Chicken pancreas....	3.0	0.0091
Potato....	38.6	0.055

\* Expressed as equivalent to folic acid of potency 40,000.

that the enzymes lost activity during the reaction. In these cases the velocities were calculated from the active material liberated in the first 30 minute period of incubation. Shorter incubation times were not practicable.

The initial velocities of the reactions were subjected to analysis and

treatment by the methods outlined by Lineweaver and Burk (8) and the maximum velocity,  $V_{\max}$ , and Michaelis constant,  $K_s$ , for each of the enzymes estimated graphically. Table I summarizes the estimated constants for the three enzymes. Since the microbiological potencies and the molecular weights of the substrate and of the end-products are unknown, the values for the constants are necessarily given in arbitrary units. The curves shown in Fig. 1 are the theoretical ones obtained by substituting these values into the equation of Michaelis and Menten (5). The indicated points are the analytical results.

An examination of Fig. 1 reveals that at pH 7 and at low substrate concentrations the rat liver enzyme is much more efficient than either the pancreas or potato preparations. These results are in agreement with our observation that much more of the pancreas enzyme is required for satisfactory determination of potential *Streptococcus faecalis*-stimulating factor in some materials.

It should be pointed out that the highest substrate concentrations available to us did not permit a satisfactory determination of  $K_s$  for the potato enzyme; the value given is therefore to be regarded as an approximate figure.

The possible physiological rôle of these enzymes will be discussed in a later paper.

#### SUMMARY

The method for preparing a rat liver enzyme capable of converting inactive precursors to *Streptococcus faecalis*-stimulating factor has been improved and the preparation of a similar enzyme from potatoes is described.

The Michaelis constants at pH 7 and 32° of the rat liver enzyme and similar enzymes from potatoes and chicken pancreas were found to be 0.29, 38.6, and 3.0 respectively. The values are given in terms of mg. of material equivalent to folic acid of potency 40,000.

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# A STUDY OF ENZYMIC REACTIONS CATALYZED BY PIGEON LIVER EXTRACTS\*

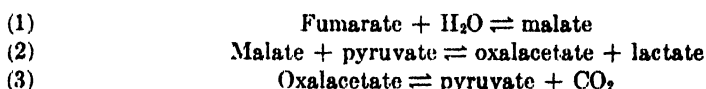
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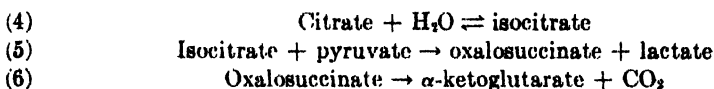
Pigeon liver is the only vertebrate tissue from which cell-free extracts capable of converting carbon dioxide into organic combination have been prepared (1). A further study of extracts from this tissue has revealed, as was expected, the presence of enzyme systems capable of catalyzing other chemical transformations. The present report is concerned with three reactions occurring in these extracts.

I. The coupled reaction between pyruvate and malate which is responsible for the fixation of carbon dioxide in cell-free extracts of pigeon liver has been investigated in more detail. As previously reported (1), the sequence may be summarized by the equations



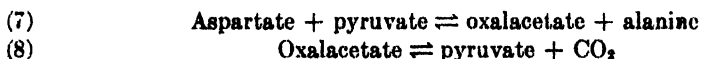
For convenience, this sequence will be referred to in the text as the reaction between pyruvate and malate.

II. A hitherto undescribed reaction between pyruvate and isocitrate to yield  $\alpha$ -ketoglutarate and lactate has been observed. The mechanism of this reaction is believed to be analogous to that of the reaction between pyruvate and malate.



This set of reactions will be referred to as the reaction between pyruvate and isocitrate.

III. A transamination between pyruvate and aspartate has been observed and studied. The reaction mechanism is as follows:



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The study of these reactions presents the following points of interest. (1) An enzymic reaction between pyruvate and isocitrate has not been described previously. (2) The reactions between pyruvate and malate and between pyruvate and isocitrate are accelerated by the presence of *either* pyridine nucleotide, diphosphopyridine nucleotide (DPN), or triphosphopyridine nucleotide (TPN). This is in marked contrast to the specific relationship between these coenzymes and the lactic (2), malic (2), and isocitric (3) dehydrogenases found in extracts of other tissues. (3) Evidence has been obtained which suggests that in pigeon liver extracts the decarboxylation of oxalosuccinate (Equation 6) is catalyzed by an enzyme similar to oxalacetate carboxylase (1). This is in contrast to the accepted view that oxalosuccinate, the hypothetical primary oxidation product of isocitrate, is spontaneously decarboxylated to  $\alpha$ -ketoglutarate (4, 3). (4) The transamination of pyruvate with aspartate in pigeon liver extracts is catalyzed by an enzyme which appears to differ in physical properties from the transaminases previously described.

### Methods

*Enzyme Preparations*—The pigeon liver extracts used in these experiments were prepared in the same manner as those previously employed in the study of carbon dioxide fixation (1). Pigeon liver acetone powder was extracted for 10 minutes with 8 parts of water, and the supernatant, after centrifugation, was dialyzed for 12 to 72 hours at 2° against 0.025 M phosphate, pH 7.4. Such a preparation contains fumarase, aconitase, oxalacetate carboxylase, and the various dehydrogenases described in the text.

Extracts of pigeon breast muscle were prepared from pigeon breast muscle acetone powder in the same manner.

*Preparation and Assay of Pyridine Nucleotide Coenzymes*—Diphosphopyridine nucleotide (DPN) was prepared from Fleischmann's bakers' yeast by the method of Williamson and Green (5). The purity of the preparation was 45 per cent as determined by hydrosulfite reduction (6).

This value was checked by use of a DPN assay method based on the glycolytic system used by Speck and Evans (7) in the study of the effect of quinine and atabrine upon the 3-phosphoglyceraldehyde dehydrogenase of rabbit skeletal muscle. Carbon dioxide production in this system is a function of the concentration of DPN. When DPN is added in amounts up to 50  $\gamma$  per vessel, the relation between carbon dioxide evolution and DPN concentration is almost linear. Carbon dioxide production in the absence of added DPN is very small. The dissociation constant of the protein-coenzyme complex was found to be  $3.0 \times 10^{-4}$  mole of DPN per liter for the 3-phosphoglyceraldehyde dehydrogenase of rabbit skeletal muscle.

The agreement of this value with the dissociation constant of  $3.16 \times 10^{-5}$  mole of DPN per liter obtained by Warburg and Christian (8) for yeast 3-phosphoglyceraldehyde dehydrogenase is an indication of the validity of the assay method. No TPN was found in the DPN preparation when assayed according to Haas (9).

Part of the triphosphopyridine nucleotide (TPN) used in these experiments was a gift of Dr. Erwin Haas. Most of the work, however, was performed with a TPN preparation made by the method of Warburg, Christian, and Griese (10) as modified by Altman (11). Assay of this preparation gave a TPN content of 10 per cent (9). Its DPN content was 0.4 per cent as determined by the method outlined above.

The concentration of DPN and TPN is expressed throughout the paper in terms of the pure coenzyme as determined by appropriate assay.

*Manometric Methods*—Since the reactions studied in this paper all result in the liberation of carbon dioxide, manometric determination of the carbon dioxide evolution at pH 5.5 to 6.0 affords a convenient method of following the course of the reactions. An acetate buffer of pH 5.0 was added to the reaction mixtures in amounts indicated in individual experiments, and the pH of the final mixture was always between pH 5.5 and 6.0. The usual Warburg manometric technique was employed. The bath temperature was 40°, and the gas phase was air, N<sub>2</sub>, or 95 per cent N<sub>2</sub>-5 per cent CO<sub>2</sub>. Frequent control experiments indicated that the oxygen consumption of the enzyme preparations was negligible at all times.<sup>1</sup> The substrates were added as neutral sodium or potassium salts tipped in from the side arms.

*Thunberg Experiments*—The Thunberg methylene blue technique was used to test the coenzyme specificity of lactic, malic, and isocitric dehydrogenases. The yeast flavoprotein, which is a component of the isocitrate system, was prepared according to the procedure of Warburg and Christian (13) up to the point of methanol precipitation. The glucose-6-phosphate system was used to study the possible conversion of DPN to TPN. For this purpose, hexose-6-phosphate and glucose-6-phosphate dehydrogenase were prepared according to the directions of Warburg and Christian (14).

*Analytical Methods*—Pyruvate was determined either by the carboxylase method (15) or by the colorimetric method of Friedemann and Haugen (16). Lactate was measured according to Barker and Summerson (17).  $\alpha$ -Ketoglutarate was determined by the method of Friedemann and Haugen (16). Citrate was determined by the pentabromoacetone method of Perl-

<sup>1</sup> After an incubation of several hours, pigeon liver extracts consume oxygen in the presence of  $\alpha$ -ketoglutarate. A reaction similar to the formation of malonate from oxalacetate in pig heart extracts (Vennesland and Evans (12)) is probably responsible for this delayed oxygen uptake.



man, Lardy, and Johnson (18), and the ninhydrin method of Hamilton and Van Slyke (19) was used for the estimation of  $\alpha$ -amino nitrogen.

### *I. Reaction between Pyruvate and Malate*

When dialyzed pigeon liver extract, together with  $Mn^{++}$  and DPN, is incubated with pyruvate and fumarate, carbon dioxide is evolved in quantities equal to the fumarate added (Fig. 1). The carbon dioxide evolution

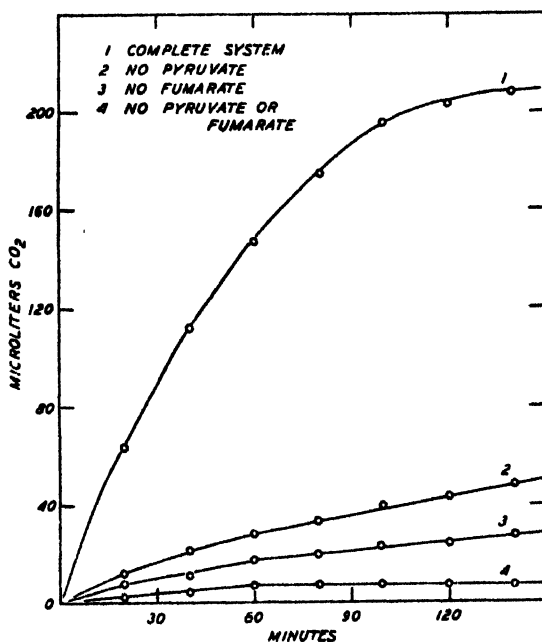


FIG. 1. The reaction between pyruvate and malate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, at pH 5.0, 0.002 M  $MnCl_2$ ,  $5.0 \times 10^{-5}$  M diphosphopyridine nucleotide, 0.004 M sodium fumarate (224 microliters), and 0.02 M sodium pyruvate (1120 microliters) in a volume of 2.4 ml. The temperature was 39°, and the gas phase was air.

is very small when either pyruvate or fumarate alone serves as a substrate, the amount obtained varying with different enzyme preparations. The behavior of *l*-malate is identical with that of fumarate, indicating that the enzyme contains an active fumarase.

These facts are in accord with the conclusion (1) that the reaction between pyruvate and malate occurs by the mechanism summarized in Equations 1, 2, and 3. The net reaction consists of a conversion of fumarate to lactate and carbon dioxide.

(9)



*Effect of Pyruvate Concentration*—Although the pyruvate concentration does not change during the reaction, the acid being reformed in an amount equal to the quantity used, the participation of pyruvate in the reaction is reflected by the effect of pyruvate concentration on the reaction rate (Fig. 2).

*Effect of Cofactors*—The addition of pyruvate and fumarate to undialyzed enzyme preparations gives rise to a rapid evolution of carbon dioxide. After dialysis of the enzyme preparations, the rate of reaction between the

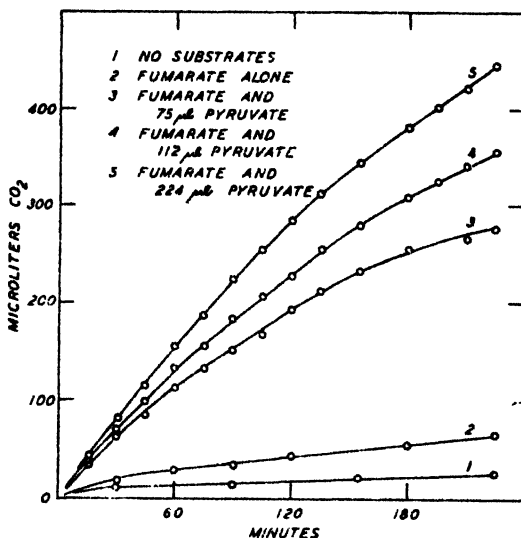


FIG. 2. The effect of pyruvate concentration on the reaction between pyruvate and malate. Vessel 1 contained 2.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.0004  $\text{MnCl}_2$ ,  $3.0 \times 10^{-5}$  M diphosphopyridine nucleotide, and water to 3.7 ml. All the other vessels contained in addition 0.0135 M sodium fumarate (1120 microliters) and sodium pyruvate in the amounts indicated above. The temperature was  $39^\circ$ , and the atmosphere was 5 per cent  $\text{CO}_2$  95 per cent  $\text{N}_2$ .

two substrates becomes very slow. It can be restored, however, by the addition of a boiled tissue extract.<sup>2</sup> The effect of the extract can be reproduced by the addition of  $\text{Mn}^{++}$  ions and DPN or TPN (Fig. 3). Stimulation of carbon dioxide production is caused by  $\text{Mn}^{++}$  ions alone, and either or both dinucleotides are ineffective in their absence. However, the addition of the dinucleotides plus  $\text{Mn}^{++}$  causes a more rapid evolution of carbon dioxide than that observed in the presence of the inorganic ions alone. The

<sup>2</sup> The tissue extracts were prepared by mixing 1 part of freshly ground pigeon liver or breast muscle with 1 part of water, boiling for 5 minutes, cooling, and filtering

stimulating effect of the dinucleotides is minimal with enzyme preparations dialyzed for 12 hours and pronounced with extracts prepared by a prolonged dialysis of 48 to 72 hours. The effects of DPN and of TPN are not additive.

Since the reaction of Equation 2 is catalyzed by the lactic and malic dehydrogenases which require DPN for their action, the stimulating effect of DPN on this reaction is to be expected. However, the stimulating effect of TPN is of interest, inasmuch as the malic and lactic dehydrogenases,

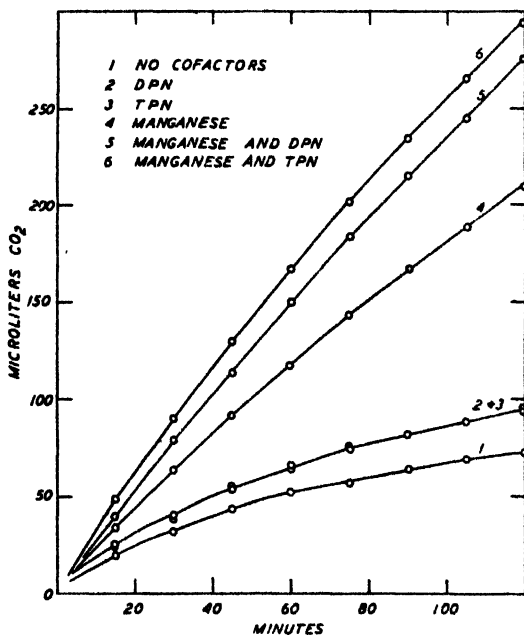


FIG. 3. The effect of cofactors on the reaction between pyruvate and malate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.02 M sodium pyruvate, and 0.02 M sodium fumarate in 2.4 ml. total volume. Cofactors were added to the other vessels as indicated above in the following concentrations: diphosphopyridine nucleotide and triphosphopyridine nucleotide,  $5.0 \times 10^{-5}$  M;  $\text{MnCl}_2$ , 0.002 M. All vessels were incubated in air at  $39^\circ$ .

when prepared from other tissues, have been found to require DPN specifically. Accordingly, an examination of the coenzyme specificities of the lactic and malic dehydrogenases of pigeon liver was carried out by the Thunberg technique. Under experimental conditions in which the coenzymes were present in concentrations less than that required for a maximum rate of methylene blue reduction, so that the rate of reduction of the dye was a function of the quantity of coenzyme present, it was found that the lactic and malic dehydrogenases of pigeon liver responded to the presence of either DPN or TPN (Table I).

While the TPN preparation used in these experiments contained small quantities of DPN, the following considerations suggest that the observed effects were due to TPN itself. Kubowitz and Ott (20) found that the dissociation constant of the protein-coenzyme complex of the lactic dehydrogenase of rat skeletal muscle was  $6 \times 10^{-6}$  mole of DPN per liter. While the dissociation constant of the lactic dehydrogenase of pigeon liver is not known, it may reasonably be assumed that it is not less than  $10^{-6}$  mole of pyridine nucleotide per liter. This is the concentration of coenzyme which gives half the maximum reaction rate. In the experiment of Table I, TPN and DPN were added in concentrations of  $1 \times 10^{-5}$  M. In the system to which the TPN preparation was added, the concentration of DPN added in the TPN preparation was  $4 \times 10^{-7}$  M; i.e., well below the minimal concen-

TABLE I

*Coenzyme Specificity of Lactic and Malic Dehydrogenases in Extracts from Pigeon Tissues*

The experiments were conducted at 25° by the Thunberg technique. System (1) consisted of 1.0 ml. of dialyzed extract, 0.02 M phosphate buffer, pH 7.4, 0.15 M NaCN, pH 7.4, and 0.1 M lithium lactate or sodium malate, all in a volume of 2.5 ml. At 0 time, 0.1 ml. of 0.1 per cent methylene blue was added from the cap.

System No.	Reduction time		
	Malic dehydrogenase	Lactic dehydrogenase	
	Pigeon liver extract	Pigeon liver extract	Pigeon breast muscle extract
	min.	min.	min.
1. As described above	35	36	60
2. Plus $1 \times 10^{-5}$ M diphosphopyridine nucleotide	12	10	6
3. " $1 \times 10^{-5}$ " triphosphopyridine "	14	25	17

tration giving half the maximum reaction rate. Yet the effect of TPN upon the reduction of methylene blue was from one-third to almost equal the effect of DPN. These results find their simplest explanation in the assumption that TPN as well as DPN forms an active enzyme-coenzyme complex with the lactic and malic dehydrogenases of pigeon tissues.

The effect of TPN upon lactate oxidation may also be explained by postulating the presence of an enzyme system in pigeon liver extracts capable of the rapid transformation of TPN into DPN, a reaction known to occur in yeast extracts (21). However, unpublished work by Dr. L. E. Montoya (Rockefeller Foundation Fellow) makes the occurrence of such a transformation seem improbable. Working in this laboratory, he obtained a 20-fold purification of the lactic dehydrogenase of pigeon breast muscle.

At this stage of purity, the dehydrogenase showed the same relative activity toward DPN and TPN as did the original extract. It seems unlikely that both the lactic dehydrogenase and an enzyme converting TPN into DPN would have been equally fractionated throughout the purification procedure.

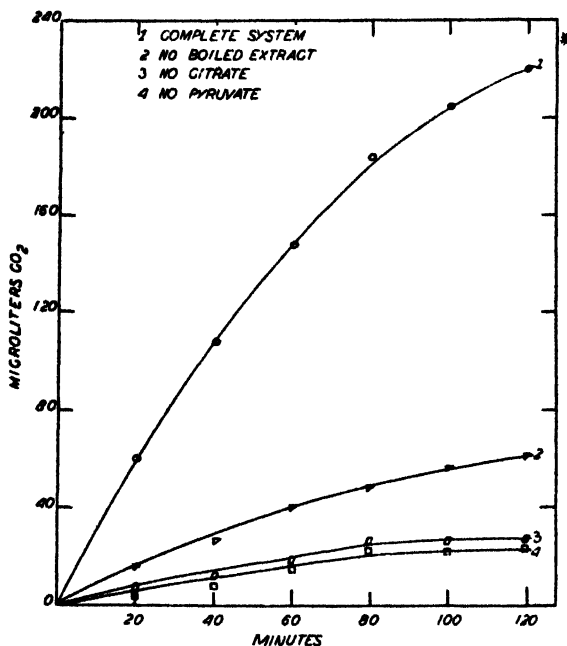


FIG. 4. The reaction between pyruvate and isocitrate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 1.0 ml. of boiled extract of pigeon breast muscle, 0.05 M acetate buffer, pH 5.0, 0.0025 M potassium citrate (224 microliters), and 0.005 M sodium pyruvate (448 microliters). The volume was 4 ml. The bath temperature was 40°, and the gas phase was 5 per cent CO<sub>2</sub>-95 per cent N<sub>2</sub>.

## II. Reaction between Pyruvate and Isocitrate

In addition to the reaction between pyruvate and malate, pigeon liver extracts catalyze an analogous reaction between pyruvate and isocitrate. When pyruvate and citrate, together with the necessary cofactors, are incubated with pigeon liver extracts, carbon dioxide is produced in an amount equivalent to the substrate present in the smaller concentration. The carbon dioxide evolution is much less in the absence of either pyruvate or citrate or of the cofactors. A typical experiment in which the cofactors were furnished in the form of a boiled tissue extract is shown in Fig. 4.

*Effect of Substrate Concentration*—The optimal concentration of citrate is

about 0.0025 M. Citrate concentrations higher than this may be inhibitory and with 0.025 M citrate the reaction between pyruvate and isocitrate is almost completely inhibited. With 0.0025 M citrate, variation of the pyruvate concentration from 0.0025 to 0.0125 M does not alter appreciably the rate of the reaction.

*Effect of Cofactors*—The effect of boiled tissue extract on the dialyzed enzyme can be quantitatively reproduced by  $Mn^{++}$ , DPN, and TPN. As

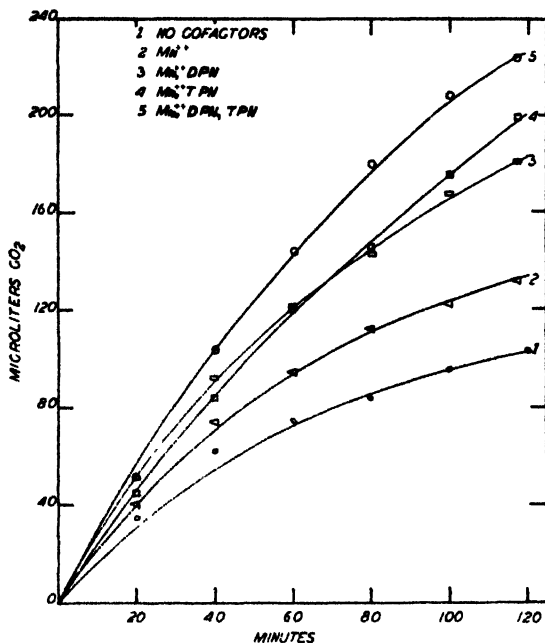


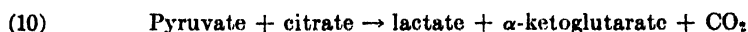
FIG. 5. The effect of cofactors on the reaction between pyruvate and isocitrate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.0025 M potassium citrate (224 microliters), and 0.005 M sodium pyruvate (448 microliters). The cofactors were added to the other vessels as indicated, in the following concentrations: diphosphopyridine nucleotide and triphosphopyridine nucleotide,  $3.0 \times 10^{-5}$  M;  $MnCl_2$ , 0.005 M. The volume was 4.0 ml., the temperature  $40^\circ$ , and the gas phase 5 per cent  $CO_2$ -95 per cent  $N_2$ .

in the reaction between pyruvate and malate,  $Mn^{++}$  alone causes a large increase in the reaction rate; DPN and TPN significantly accelerate the reaction between pyruvate and isocitrate only in the presence of  $Mn^{++}$  (Fig. 5). In the experiment of Fig. 5 neither coenzyme was present in optimal concentration and the effects appear to be additive, but when DPN and TPN are added in larger amounts, no additive effect is noted.

To assure an adequate concentration of  $Mn^{++}$ ,  $MnCl_2$  was routinely

added in amounts larger than the quantity of citrate added, since  $Mn^{++}$  forms an un-ionized and inactive complex with citrate (22). The inhibitory effect of high citrate levels on the reaction between pyruvate and isocitrate is probably due to the combination of citrate with  $Mn^{++}$ .

*Nature of Reaction between Pyruvate and Isocitrate*.—Analysis of initial reactants and final products in the reaction between pyruvate and isocitrate showed that for every mole of pyruvate and of citrate disappearing 1 mole each of lactate,  $\alpha$ -ketoglutarate, and carbon dioxide was formed (Table II). The equation for the net reaction is



From the work of Martius (23), Johnson (24), and Adler *et al.* (3) it appears probable that the first step in the reaction is the transformation of citrate to

TABLE II

*Chemical Changes Occurring during Reaction between Pyruvate and Isocitrate*

2.0 ml. of pigeon liver extract were incubated at 40° in a reaction mixture of 4.0 ml. volume which contained 0.005 M  $MnCl_2$ ,  $3 \times 10^{-5}$  M diphosphopyridine nucleotide and triphosphopyridine nucleotide, and 0.05 M acetate buffer, pH 5.0. The gas phase was 5 per cent  $CO_2$ -95 per cent  $N_2$ , and the incubation period was 1 hour. The substrates were added from the side arm in the amounts indicated below. (The pyruvate values are not corrected for interference on the part of  $\alpha$ -ketoglutarate.)

The results are expressed in micromoles.

	Pyruvate	Citrate	Lactate	$\alpha$ -Keto-glutarate	$CO_2$
Initial . . . . .	11.0	6.7	0.6	0.0	0.0
Final . . . . .	6.5	0.4	5.9	5.6	5.0
$\Delta$ . . . . .	-4.5	-6.3	+5.3	+5.6	+5.0

isocitrate by the enzyme aconitase. Isocitrate then reacts with pyruvate and yields lactate and oxalosuccinate (Equation 5). The latter is then decarboxylated to  $\alpha$ -ketoglutarate and carbon dioxide (Equation 6).

In the data presented in Table II, lactate was determined by the Barker and Summerson method (17) (in which a copper lime reagent is used for the partial removal of pyruvate). This was considered specific for lactate and actual isolation of lactic acid was regarded as unnecessary. However, the colorimetric procedure for  $\alpha$ -ketoglutarate (16) is not specific, and the presence of this substance was confirmed by its isolation as the 2,4-dinitrophenylhydrazone. A 100 ml. Warburg vessel was filled with 20 ml. of pigeon liver extract, 20 ml. of boiled extract of pigeon breast muscle, 2.0 ml. of M acetate buffer, pH 5.0, 2.0 ml. of 0.1 M  $MnCl_2$ , 2.0 ml. of 0.1 M citrate, and 2.0 ml. of 0.1 M pyruvate. After an incubation period of 90 minutes under 5 per cent  $CO_2$ -95 per cent  $N_2$  at 40°, the theoretical amount

of carbon dioxide had been evolved. To remove traces of pyruvate, 0.5 ml. of a yeast carboxylase preparation was added. After 5 minutes the solution was deproteinized by the addition of 0.2 volume of 20 per cent  $\text{HPO}_3$ , centrifuged, and aerated to remove acetaldehyde. The filtrate was concentrated to 10 ml. under reduced pressure, and 10 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl were added. The hydrazone separated out immediately and, after 2 hours in the ice box, the precipitate was filtered off, dried, and weighed. 31 mg. of a substance melting at  $206\text{--}207^\circ$  (corrected) were obtained (50 per cent of the quantity of  $\alpha$ -ketoglutaric acid 2,4-dinitrophenylhydrazone demanded by theory). After two recrystallizations from 60 per cent ethanol, 14 mg. of the hydrazone were obtained which melted at  $215^\circ$  (corrected). An authentic sample of  $\alpha$ -ketoglutaric acid 2,4-dinitrophenylhydrazone prepared and recrystallized in the same manner also melted at  $215^\circ$  (corrected). The melting point of a mixture of the two substances showed no depression. Further recrystallization from ethyl acetate and from hot water did not raise the melting point of either sample. When citrate was omitted from the reaction mixture,  $\alpha$ -ketoglutaric acid 2,4-dinitrophenylhydrazone could not be found.

*Mechanism of Reaction between Pyruvate and Isocitrate*—If lactic dehydrogenase is specific for DPN and isocitric dehydrogenase is specific for TPN, it is not possible to formulate a mechanism for the direct transfer of hydrogen between pyruvate and isocitrate involving mediation by the pyridine nucleotide coenzymes alone. However, the assumption that both lactic and isocitric dehydrogenases are active with either DPN or TPN offers a plausible mechanism for hydrogen transfer between the two substrates. According to this hypothesis, either coenzyme can transfer hydrogen between pyruvate and isocitrate.

Direct test of the coenzyme specificities of the two dehydrogenases involved supported this view. As has already been shown in Table I, lactic dehydrogenase is activated by both DPN and TPN. Table III presents data which show that the isocitric dehydrogenase of pigeon liver is also active with both DPN and TPN. The approximately equal effects of DPN and TPN upon isocitrate oxidation, together with the low concentration of TPN in the DPN preparation, indicate that DPN itself can activate the isocitric dehydrogenase of pigeon liver. However, the possibility that DPN is converted into TPN was also investigated, with the glucose-6-dehydrogenase system as a test system for the formation of TPN. In previous demonstrations of the conversion of DPN to TPN in yeast (25), adenosine triphosphate was a component of the system, but since the pyruvate-isocitrate reaction proceeds in dialyzed extracts in the absence of adenosine triphosphate, none was added here. To determine the ability of the pigeon



liver extract to transform DPN into TPN, the extract was incubated with DPN, hexose-6-phosphate, glucose-6-phosphate dehydrogenase, and yeast flavoprotein. After incubation under these conditions, the rate of reduc-

TABLE III

*Coenzyme Specificity of Isocitric Dehydrogenase in Extracts of Pigeon Tissues*

Thunberg experiment; 25°. System (1) consisted of 0.3 ml. of extract, 0.3 ml. of yeast flavoprotein (2.5  $\gamma$  of bound flavin per ml.), 0.0025 M phosphate, pH 7.4, 0.01 M potassium citrate, and 0.003 M  $\text{MnCl}_2$ . The total volume was 2.0 ml. At 0 time, 0.1 ml. of 0.2 per cent methylene blue was tipped in.

System No.	Reduction time	
	Pigeon liver extract	Pigeon breast muscle extract
	min.	min.
1. As described above.....	55	60
2. Plus $8 \times 10^{-5}$ M diphosphopyridine nucleotide.....	18	21
3. " $8 \times 10^{-5}$ " triphosphopyridine ".....	25	30
4. As (2), but no added flavoprotein.....	>120	>120
5. " (3), " " " ".....	>120	>120

TABLE IV

*Failure of Pigeon Liver Extracts to Convert Diphosphopyridine Nucleotide to Triphosphopyridine Nucleotide*

The Thunberg experiments were carried out at 25° in a volume of 3.0 ml. System (1) consisted of 0.1 ml. of glucose-6-phosphate dehydrogenase, 0.3 ml. of yeast flavoprotein (2.5  $\gamma$  of bound flavin per ml.), 0.025 M phosphate buffer, pH 7.4, 0.01 M sodium hexose-6-phosphate, and  $4 \times 10^{-5}$  M triphosphopyridine nucleotide. 0.1 ml. of 0.1 per cent methylene blue was added from the cap. The pigeon liver extract was incubated with the rest of the system for 15 minutes in air and 15 minutes *in vacuo* before the methylene blue was tipped in.

System No.	Reduction time
	min.
1. Complete.....	2
2. No triphosphopyridine nucleotide.....	70
3. $4 \times 10^{-5}$ M diphosphopyridine nucleotide.....	45
4. $4 \times 10^{-5}$ " " " and 1.0 ml. pigeon liver extract.....	35
5. $4 \times 10^{-5}$ M diphosphopyridine nucleotide and 1.0 ml. heat-inactivated pigeon liver extract.....	30

tion of added methylene blue was measured. An increase in the rate of methylene blue reduction after incubation with pigeon liver extract would indicate the conversion of DPN into TPN. When tested in this manner (Table IV), both pigeon liver extract and partially purified oxalacetate carboxylase had no effect which could not be duplicated by the same

amount of heat-inactivated enzyme. The slight acceleration of methylene blue reduction was probably due to traces of TPN in the tissue extracts. Certainly a rapid conversion of DPN to TPN, such as would be necessary to explain the effect of DPN upon the oxidation of isocitrate in a system with pyruvate or with flavoprotein and methylene blue, was not observed.

However, other tissues, notably pigeon breast muscle, also show a similar lack of coenzyme specificity on the part of their lactic and isocitric dehydrogenases (Tables I and III). One might expect, therefore, that in such preparations the reaction between pyruvate and isocitrate should occur, since the enzyme aconitase is known to be widely distributed, and since the decarboxylation of oxalosuccinate is considered to occur spontaneously. Nevertheless, aqueous extracts of acetone powders of beef liver, pig liver, rabbit liver, pig heart, pig kidney, and pigeon breast muscle do not catalyze the reaction between pyruvate and isocitrate. Nor do they catalyze the reaction between pyruvate and malate. Since the latter reaction is inhibited by small amounts of oxalacetate which accumulate, it appears certain that the difference between extracts of pigeon liver and those of other tissues with respect to their ability to catalyze the reaction between pyruvate and malate lies in the presence of oxalacetate carboxylase in the pigeon liver extracts. An analogous inhibition of the reaction between pyruvate and isocitrate is not possible if the decarboxylation of oxalosuccinate is completely spontaneous. If it were assumed, however, that pigeon liver contains an enzyme (similar to oxalacetate carboxylase) capable of decarboxylating oxalosuccinate, the absence of this enzyme in other tissue extracts would then explain why the reaction between pyruvate and isocitrate occurs only in pigeon liver extracts. Since free oxalosuccinic acid has never been prepared, a direct examination of the rate of its spontaneous decarboxylation and of the effect of pigeon liver extract upon the process is impossible.

*Effect of Partially Purified Oxalacetate Carboxylase upon Reaction between Pyruvate and Isocitrate in Extracts of Pigeon Breast Muscle*—The hypothesis outlined in the previous section was tested indirectly, therefore, by adding to pigeon breast muscle extract a preparation of partially purified oxalacetate carboxylase<sup>3</sup> free from lactic and isocitric dehydrogenases. Although

<sup>3</sup> Evans, E. A., Jr., Francis, A. M., and Vennesland, B., unpublished results. The preparations were made from the usual pigeon liver extracts by slowly adding methanol at 0° until the concentration was 50 per cent. The precipitate was centrifuged off and discarded. The supernatant was poured into an equal volume of 0.1 M acetate buffer, pH 5.0. The precipitate was centrifuged off, redissolved in 0.025 M phosphate, pH 7.4, and dialyzed against the same buffer. Such preparations contain oxalacetate carboxylase, traces of lactic dehydrogenase, and no isocitric dehydrogenase.

incapable of catalyzing the reaction alone, this preparation caused the reaction between pyruvate and isocitrate to occur in pigeon breast muscle extracts (Fig. 6). The reaction taking place under these circumstances was identical with that which occurs in pigeon liver extracts. The presence of both pyruvate and citrate, as well as of  $Mn^{++}$  and the pyridine nucleotide coenzymes, is necessary for its occurrence. Heating of the oxalacetate carboxylase preparation causes loss of its ability to promote the reaction between pyruvate and isocitrate in pigeon breast muscle extracts. The

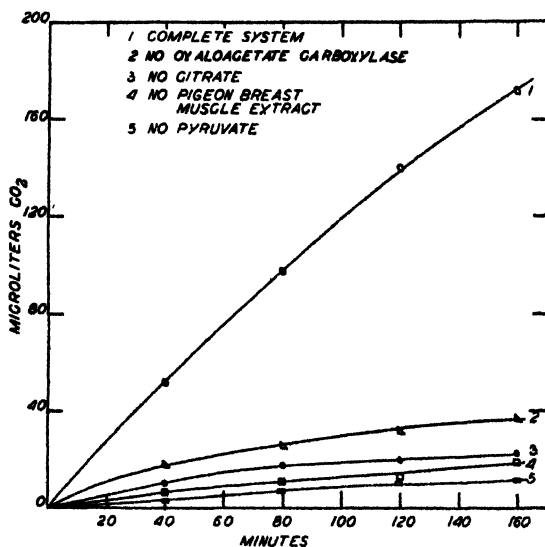


FIG. 6. The effect of partially purified oxalacetate carboxylase on the reaction between pyruvate and isocitrate in pigeon breast muscle extracts. Vessel 1 contained 1.0 ml. of dialyzed pigeon breast muscle extract, 0.5 ml. of partially purified oxalacetate carboxylase, 0.05 M acetate buffer, pH 5.0, 0.005 M  $MnCl_2$ ,  $3.0 \times 10^{-5}$  M diphosphopyridine nucleotide,  $3.0 \times 10^{-5}$  M triphosphopyridine nucleotide, 0.0025 M potassium citrate, and 0.005 M sodium pyruvate, all in a volume of 4.0 ml. All vessels were incubated at  $40^\circ$  under 5 per cent  $CO_2$ -95 per cent  $N_2$ .

ability of partially purified oxalacetate carboxylase to catalyze the pyruvate-isocitrate reaction in pigeon breast muscle extracts roughly parallels its oxalacetate carboxylase activity.

These results indicate that pigeon liver extracts contain at least one heat-labile factor other than lactic and isocitric dehydrogenases which is essential for the occurrence of the coenzyme-linked reaction between pyruvate and isocitrate. The experimental evidence suggests that this factor is an enzyme which decarboxylates oxalosuccinate, either oxalacetate carboxylase or an enzyme similar to it.

Since di- and trivalent cations accelerate the decarboxylation of oxalacetate, it might be expected that no enzyme would be necessary for the decarboxylation of oxalacetate and that the reaction between pyruvate and malate would proceed in the absence of oxalacetate carboxylase. However, even in the presence of high concentrations of  $Mn^{++}$  ion (0.002 to 0.02 M), no reaction between pyruvate and malate is observed in the absence of oxalacetate carboxylase. Similarly,  $Mn^{++}$  ion does not catalyze the reaction between pyruvate and isocitrate, even in the presence of the proper cofactors and dehydrogenases, unless an additional heat-labile substance from pigeon liver is also present.

On the basis of the evidence outlined above, we believe that the reaction between pyruvate and isocitrate occurs in the following manner. The primary step in the reaction, the oxidation-reduction between pyruvate and isocitrate (Equation 5), is mediated by both DPN and TPN, each of which reacts with both dehydrogenase systems. If oxalosuccinate formed by the oxidation of isocitrate is not removed immediately, it then inhibits the oxidation of isocitrate in the same manner that oxalacetate inhibits the oxidation of malate (26). In pigeon breast muscle, the oxidation-reduction step proceeds readily, but the enzyme which decarboxylates oxalosuccinate is absent, and this product is removed only by non-enzymic decarboxylation. The rate of spontaneous decarboxylation is not sufficient to keep the concentration of oxalosuccinate below the level at which it inhibits the oxidation of isocitrate, and the reaction between pyruvate and isocitrate does not occur. In pigeon liver, an enzyme similar to oxalacetate carboxylase decarboxylates oxalosuccinate at such a rapid rate that its concentration in the reaction mixture does not reach an inhibitory level, and the pyruvate-isocitrate reaction goes to completion.

*Oxidation of Isocitrate by Yeast Flavoprotein and Methylene Blue*—However, this mechanism does not explain why isocitrate is oxidized by pigeon breast muscle extracts in the presence of yeast flavoprotein and methylene blue (3) (Table III), although isocitrate is not oxidized by these extracts in the presence of pyruvate. We have confirmed the observation of Adler *et al.* (3) that crude yeast flavoprotein is necessary for the oxidation of isocitrate by methylene blue (Table III). Since the concentration of the flavoprotein which reoxidizes reduced TPN was low in their preparation, these workers reasonably assumed that the function of the added yeast flavoprotein (which reoxidizes both TPN and DPN) was to reoxidize the TPN reduced by isocitrate.



However, it is difficult to apply this explanation to the results obtained with extracts of pigeon tissues. In these extracts, yeast flavoprotein is required

for the reduction of methylene blue in the presence of isocitrate when either DPN or TPN is present (Table III). Yet with lactate or malate as the substrate, no added flavoprotein is necessary (Table I). If it is assumed that the only function of the yeast flavoprotein is as a mediator in hydrogen transport between the pyridine nucleotides and methylene blue, then it must be concluded that the coenzymes reduced by isocitrate differ in some manner from the coenzymes reduced by lactate or malate. Since such a

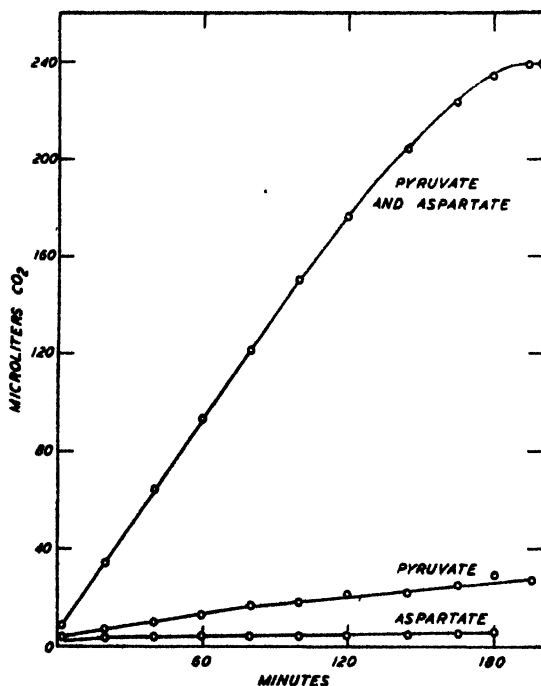


Fig. 7. The reaction between pyruvate and aspartate. All vessels contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.002 M  $\text{MnCl}_2$ , and water to a volume of 2.2 ml. 0.0045 M sodium *l*-aspartate (224 microliters) and 0.0225 M sodium pyruvate (1120 microliters) were added as indicated above. The temperature was 39° and the atmosphere was air.

conclusion is unsatisfactory, we feel that in extracts of pigeon liver and breast muscle crude yeast flavoprotein plays some rôle in the oxidation of isocitrate by methylene blue other than that of a simple mediator, but we are at present unable to suggest what such an additional rôle might be. Detailed knowledge of the mechanism of isocitrate oxidation in the systems with pyruvate and with flavoprotein and methylene blue will undoubtedly resolve the apparent conflict between the two sets of observations.

### III. Reaction between Pyruvate and Aspartate

When pigeon liver extract is incubated with pyruvate and *l*-aspartate, a quantity of carbon dioxide is evolved equal to the aspartate added (Fig. 7). The reaction requires  $Mn^{++}$ , but DPN and TPN have no effect on the reaction rate (Fig. 8). These results can be explained by postulating a transamination of pyruvate with aspartate to form alanine and oxalacetate,

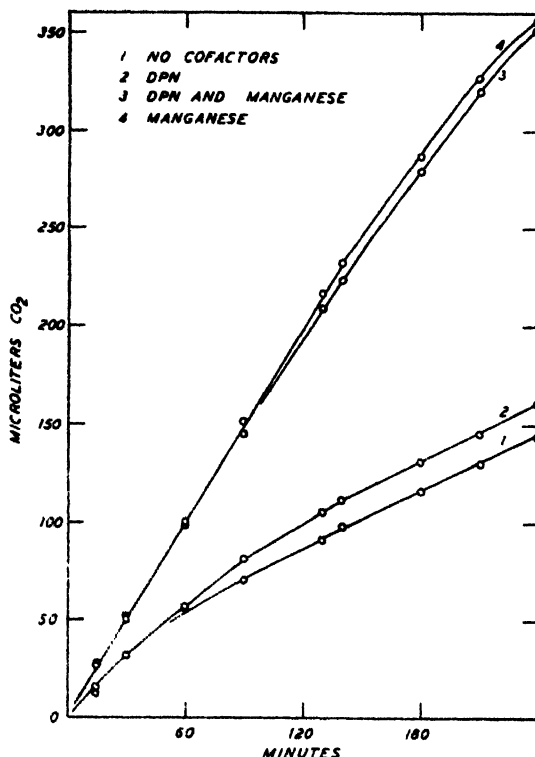


FIG. 8. The effect of cofactors on the reaction between pyruvate and aspartate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.02 M sodium pyruvate, and 0.008 M sodium *l*-aspartate in 2.4 ml.  $5.0 \times 10^{-5}$  M diphosphopyridine nucleotide and 0.002 M  $MnCl_2$  were added to the other vessels as indicated. All were incubated in air at  $39^\circ$ .

the latter then being decarboxylated by oxalacetate carboxylase (Equations 7 and 8). The equation for the net reaction would be



The validity of Equation 12 was tested by analyzing for pyruvate and by measuring the carbon dioxide liberated by treatment with ninhydrin.

While all other  $\alpha$ -amino acids yield 1 mole of carbon dioxide when treated with ninhydrin, aspartate forms an unstable aldehyde which spontaneously decarboxylates to liberate an additional molecule of carbon dioxide (27). Therefore, if Equation 12 is the correct representation of the reaction between pyruvate and aspartate, at the end of the reaction the carbon dioxide liberated by ninhydrin should be half the initial value, while the pyruvate concentration should be unaltered. The data of Table V indicate that this is indeed the case and thus furnish evidence for the mechanism postulated for the reaction between pyruvate and aspartate.

TABLE V

*Chemical Changes Occurring during Reaction between Pyruvate and Aspartate*

The reaction mixture consisted of 1.5 ml. of pigeon liver extract, 0.002 M  $MnCl_2$ , 0.06 M acetate buffer, pH 5.0, 20 micromoles of sodium pyruvate, and 10 micromoles of sodium *l*-aspartate. The volume was 2.8 ml. After 4 hours incubation at 39° in an atmosphere of 5 per cent  $CO_2$ -95 per cent  $N_2$ ,  $CO_2$  evolution ceased, and the analyses were performed. The values below are corrected for the slow disappearance of pyruvate in the absence of aspartate (1 micromole per hour) and for the formation of  $\alpha$ -amino nitrogen in the absence of any added substrate (1.5 micromoles per hour).

The results are expressed in micromoles.

	Pyruvate	Ninhydrin carboxyl $CO_2$	$CO_2$
Initial .....	20.0	20.0	0.0
Final .....	19.4	9.1	8.9
$\Delta$ .....	-0.6	-10.9	+8.9

## DISCUSSION

*Role of Oxalacetate Carboxylase in Coupled Reactions*—In addition to the coupled enzymic reactions described in the present report, reactions between  $\alpha$ -ketoglutarate and fumarate and between  $\alpha$ -ketoglutarate and citrate have also been observed. These reactions are slower in rate and have not been studied in detail. The interaction between the various pairs of substrates can be explained by a general reaction scheme involving an oxidation-reduction or a transamination followed by the decarboxylation of a  $\beta$ -keto acid, either oxalacetate or oxalosuccinate.

The enzymic decarboxylation of oxalacetate by oxalacetate carboxylase can be demonstrated directly (1). Although a direct test is not possible in the case of oxalosuccinate, the evidence presented here suggests that oxalacetate carboxylase (or a similar component of pigeon liver extract) catalyzes the decarboxylation of oxalosuccinate. Final proof for this mechanism requires preparation of free oxalosuccinic acid. The availability of

this compound would also undoubtedly be of great assistance in understanding the conflicting data resulting from studies of pyruvate and of methylene blue as the ultimate oxidizing agents for isocitrate in the pigeon breast muscle extracts.

The reaction between pyruvate and isocitrate resembles in many ways the reaction between pyruvate and malate in which carbon dioxide is fixed into organic acid carboxyl groups, but it is unknown whether carbon dioxide fixation occurs during the course of this reaction.

*Specificity of Dehydrogenases toward Pyridine Nucleotide Coenzymes*—Dehydrogenases may be divided into three groups on the basis of their reactions with the pyridine nucleotide coenzymes. One group transfers hydrogen from its substrates to DPN but not to TPN. A second group transfers hydrogen to TPN but not to DPN, and a third transfers hydrogen to both coenzymes with approximately equal ease. The 3-phosphoglycer-aldehyde dehydrogenase of yeast (28), the lactic and malic dehydrogenases of pig heart (2), and the alcohol dehydrogenase of yeast (28) and of mammalian tissues (29) are all examples of the first group. Glucose-6-phosphate dehydrogenase of yeast (30) and isocitric dehydrogenase of pig heart (3) are specific for TPN. The glucose (31) and *l*-glutamic (32) dehydrogenases of mammalian liver belong to the last group of dehydrogenases. Proper recognition of coenzyme specificity may be obscured by interconversion of the coenzymes in the crude enzyme preparations frequently used. Such an interconversion has been demonstrated in yeast by von Euler and his coworkers (21, 25).

Our work with pigeon tissues has led us to the conclusion that a larger number of dehydrogenases react with both DPN and TPN than has hitherto been thought. The lactic and isocitric enzymes of pigeon tissues on the one hand and of pig heart on the other offer another example of the occurrence in different tissues of dehydrogenases with different coenzyme specificity but with identical substrate specificity.

*Transamination in Pigeon Liver Extracts*—The presence of transaminase activity in dialyzed extracts of pigeon liver acetone powder is unexpected, since the transaminases of other tissue preparations are often destroyed by drying (33) or by dialysis (33, 34). It is probable that the transaminase of pigeon liver extracts is markedly different from those previously described. Although Braunshtein and Kritsman (35) originally reported that a wide variety of amino acids entered into reactions of transamination, Cohen (33) found that only glutamic acid, aspartic acid, and alanine undergo transamination in tissue extracts at a significant rate. It is suggested that in pigeon liver aspartate might conceivably transaminate keto acids other than those corresponding to the amino acids mentioned above, because the rapid decarboxylation of oxalacetate would drive the reaction in the direction of oxalacetate formation and the transamination of the original keto acid.



## SUMMARY

Reactions occurring in cell-free extracts of pigeon liver have been studied.

1. The reaction between pyruvate and malate, which results in the fixation of carbon dioxide has been further investigated.

2. An analogous reaction between pyruvate and isocitrate has been observed and a mechanism suggested for the reaction.

3. A transamination between pyruvate and aspartate has been described.

We wish to thank Dr. Erwin Haas and Dr. Kurt Altman of the Department of Chemistry for valuable aid and advice in the preparation and assay of triphosphopyridine nucleotide. The citrate analyses were performed by Dr. John F. Speck, and the purified oxalacetate carboxylase preparations were made by Dr. A. M. Francis.

*Addendum*—Since this paper was submitted for publication, Ochoa (36) and Ochoa and Weisz-Tabori (37) have described in brief notes the preparation of oxalosuccinic acid and its enzymic decarboxylation by extracts of pig heart. The reoxidation of reduced triphosphopyridine nucleotide in the presence of  $Mn^{++}$ ,  $CO_2$ , and  $\alpha$ -ketoglutarate indicates that, in pig heart extracts, both the oxidation of isocitrate to oxalosuccinate and the decarboxylation of oxalosuccinate to  $\alpha$ -ketoglutarate are reversible reactions. These results are in full accord with the findings presented here.

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# THE BEHAVIOR OF VOLATILE SOLUTES IN THE HILL-BALDES APPARATUS, WITH PARTICULAR REFERENCE TO ETHYL ALCOHOL

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In an earlier paper<sup>1</sup> experiments were described in which the Hill-Baldes vapor tension apparatus was applied to the quantitative determination of deuterium oxide (D<sub>2</sub>O) concentration in water. It has been considered of interest to extend the observations to the study of other volatile solutes in order to provide a more rigorous test of the theory of the apparatus, even though the accuracy of the results obtained might not be sufficiently high to compete with current analytical techniques for the solutes in question. The studies reported here are for solutions up to 4 per cent by volume of ethyl alcohol in water.

The methods employed were similar to those previously described.

Fig. 1 shows a plot of the logarithm of the galvanometer deflection *versus* the time after the deposition of the drop. The data pertinent to the experiment are tabulated in the legend. Unlike the case of D<sub>2</sub>O-H<sub>2</sub>O mixtures, the slopes of these curves diminish with time, indicating that the escape of the alcohol is more rapid at the higher concentrations. Fig. 2 gives a plot of the same curves in which they have been shifted horizontally in order to make the *maximum* deflection fall on the curve for the 2 per cent concentration. The fact that all of the remaining points fall well on a single curve indicates that the galvanometer deflection is a measure of the instantaneous concentration of alcohol in the drop.

According to the theory proposed in the earlier paper, if the loss of solute is principally determined by the diffusion of solute molecules in the gas phase, the time required for the escape of one-half of the solute (half life-time) should be given by the formula

$$T = \frac{\log_e 2}{k} = \frac{0.231 \gamma r_1^2}{D}$$

where  $k$  is the fraction of the solute escaping per unit time,  $r_1$  is the radius of the drop ( = 0.074 cm.),  $D$  is the diffusion constant of solute molecules in the gas phase, and  $\gamma$  is the ratio of the density of the pure liquid solute

<sup>1</sup> Lifson, N., Lorber, V., and Hill, E. L., *J. Biol. Chem.*, **158**, 219 (1945).

Temperature °C.	$\gamma$	$T$ sec.
25	$0.704 \times 10^4$	65
30	0.535	49
35	0.323	30

to that of its saturated vapor. The tabulation gives values of  $T$  computed from this formula. The values of  $\gamma$  and  $D$  ( $= 0.137$  sq. cm. per second) are only nominal handbook values which are not expected to be of high accuracy. From Fig. 2 a mean value of the half lifetime as observed in the experiments is about 40 seconds at  $30^\circ$ .

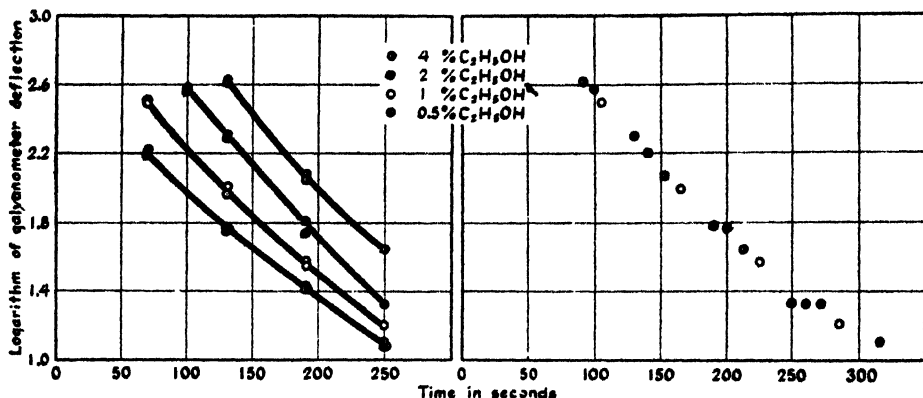


FIG. 1

FIG. 2

FIG. 1. Relationship between the logarithm of the galvanometer deflection and time after drop exposure for  $C_2H_5OH-H_2O$  mixtures up to 4 per cent  $C_2H_5OH$ . Bath temperature  $30^\circ$ ; drop size 2.02 c.mm. Calibration, 1 mm. of deflection equals 0.302 mm of NaCl per kilo of water.

FIG. 2. Plot in which the experimental points of Fig. 1 for 0.5, 1.0, and 4.0 per cent alcohol have been shifted horizontally to make the maximum deflection of each curve fall on the curve for 2.0 per cent alcohol.

The fact that the observed values of  $T$  agree well with the purely theoretical values for both  $D_2O$  and  $C_2H_5OH$  solutions, even though the absolute values for  $T$  are so different in the two cases, seems to be satisfactory evidence that the proposed theory of the apparatus is essentially correct.

#### SUMMARY

A study is reported of the behavior of drops of ethyl alcohol-water solutions in the Hill-Baldes vapor tension apparatus. Evidence is presented that (1) the galvanometer deflection is a measure of the instantaneous alcohol concentration in the drop, and (2) the rate of escape of alcohol agrees well with values predicted by a previously proposed theory of the apparatus.

# ANTIPODAL SPECIFICITY IN THE INHIBITION OF GROWTH OF LACTOBACILLUS ARABINOSUS BY AMINO ACIDS\*

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(Received for publication, April 28, 1945)

The fact that the growth of *Lactobacillus arabinosus* 17-5<sup>1</sup> can be inhibited by *d*-leucine (1) raises questions on the nature of this inhibition and its possible significance in biological synthesis of protein. Data were accordingly needed for the effects of other amino acids, especially those of the monoaminomonocarboxylic group. Such information on the comparative inhibitory effects of the enantiomorphs and racemates of alanine, valine, and leucine are here presented.

The results indicate that the test bacterium is a useful tool for studying factors which may be influencing protein synthesis within the organism. The generally regular growth results are comparable to values found with related enzyme preparations. One example of this latter type of study with *d*-amino acids is the partial blocking of histidase action by *d*-histidine (2). Edlbacher and Baur (3) have also found that the hydrolysis of *l*-leucylglycine and of glycylglycine by swine peptidase is inhibited by *d*-leucylglycine.

The peculiar experimental advantage of such an organism as *Lactobacillus arabinosus* is the possession of a complex organization which permits it readily to carry out synthesis. In this experimental framework, it is found that antipodal specificity, of the same general kind which applies to dipeptidase (4), is a principle also applicable to suspensions of intact cells of the strain of *Lactobacillus arabinosus* studied.

## Materials and Methods

*d*-Valine and *l*-Valine —The formylation procedure used for *dl*-leucine (1) was successfully applied to *dl*-valine; 40 gm. of *dl*-valine prepared (5) from Eastman practical grade isovaleric acid gave 36.5 gm. of formyl-*dl*-valine with a melting point of 141–143° and 4.8 gm. of formyl-*dl*-valine with a melting point of 139–142°. The formyl compound was resolved and hydrolyzed by the procedure of Fischer (6). 0.242 gm. of *d*-valine

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Amino acid prefixes in this paper refer solely to configuration.

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<sup>1</sup> American Type Culture Collection, No. 8014.

dissolved in 20 per cent HCl to a total weight of 20.385 gm. (specific gravity of the solution, 1.11) was examined in the polarimeter;  $[\alpha]_D = -26.6^\circ \pm 1.0^\circ$ .

*d-Alanine*—*dl*-Alanine was resolved through the benzoyl derivative by the procedure of Fischer (7). This gave *d*-alanine hydrochloride of  $[\alpha]_D = -9.7^\circ \pm 0.2^\circ$  in water, when 1.369 gm. were dissolved in water to a total weight of 12.751 gm. (sp. gr., 1.04). The hydrochloride was converted to the amino acid by solution in ethanol, chilling, and neutralization with concentrated ammonium hydroxide solution. The *d*-alanine which separated was washed with ethanol until free of chloride.

*l-Alanine*—*l*-Alanine, kindly furnished by Dr. O. K. Behrens of Eli Lilly and Company, was dissolved in hydrochloric acid solution and cleared with norit. The crystals obtained by evaporation were dissolved in ethanol and precipitated with ether. The specific rotation was  $+9.4^\circ \pm 0.2^\circ$  in water when 1.074 gm. were dissolved in water to a total weight of 12.729 gm. (sp. gr., 1.03). The *l*-alanine was regenerated from the hydrochloride in the same way as for the *d*-alanine, except that dilute lithium hydroxide solution was used instead of ammonia water.

*d-Leucine and l-Leucine*—The preparation of these isomers has been described (1).

*Microbiological Procedure*—The basal medium was that previously described (1). Growth was followed by the titration of lactic acid produced at 72 hours, as in the earlier study. Rough visual checks of growth were made in all cases.

#### EXPERIMENTAL

The comparative effects of the amino acids tested are presented in Table I. The figures are presented for one composite experiment with the nine preparations. The results are in general typical of those obtained in comparative studies of *d*, *l*, and *dl* forms of the single amino acids. For leucine, a summary of the effects of *l*-, *d*-, and *dl*-leucine is found in Table II. The corresponding figures for the valines are found in Table III. All experiments recorded in Table II or III were done at different times with different subcultures of the bacterium. All figures are the averages of duplicate determinations.

A confirmation of the different patterns of behavior of *d*-valine and *d*-leucine can be found in Fig. 1, which depicts the typical variation of growth in the presence of varying amounts of each amino acid. It is to be noted that inhibition is manifested with quantities of added *d*-amino acid much smaller than 50 mg.

The values for growth have in all cases been corrected as described previously (1). A second autoclaving effect, of interest in bioassay, has been studied. After *d*-leucine has been autoclaved with a leucine-free medium,

TABLE I

*Comparison of Amino Acid Isomers and Racemates in Inhibition of Bacterial Growth*

Addition to basal medium in tube	0.100 N acid produced		Average 0.100 N acid produced (corrected)
	ml.	ml.	ml.
None.....	2.39	2.39	2.23
50 mg. <i>l</i> -leucine.....	2.51	2.71	2.35
50 " <i>dl</i> -leucine .....	2.24	2.21	1.97
50 " <i>d</i> -leucine .....	1.54	1.48	1.25
50 " <i>l</i> -valine .....	2.56	2.53	2.17
50 " <i>dl</i> -valine.....	1.63	1.76	1.32
50 " <i>d</i> -valine .....	1.62	1.77	1.33
50 " <i>l</i> -alanine.....	2.84	3.31	2.84
50 " <i>dl</i> -alanine .....	3.05	3.04	2.81
50 " <i>d</i> -alanine .....	3.03	2.95	2.75

TABLE II

*Comparative Bacterial Growth in Presence of Various Forms of Leucine*

Experiment No.	0.100 N acid produced in control	Ratio of growth to growth in control		
		50 mg. <i>l</i> -leucine	50 mg. <i>dl</i> -leucine	50 mg. <i>d</i> -leucine
	ml.	per cent	per cent	per cent
1	2.00	106	51	16
2	2.54	92	57	39
3	3.06	86	42	33
4	2.42	62	37	27
5	1.74	98	76	58
6	2.42	87	68	44
7	2.08	107	81	64
8	1.93	104	100	70
9	2.30	80	56	22
10	2.24	105	88	56
Average..	2.27	93	66	43

TABLE III

*Comparative Bacterial Growth in Presence of Various Forms of Valine*

Experiment No	0.100 N acid produced in control	Ratio of growth to growth in control		
		50 mg. <i>l</i> -valine	50 mg. <i>dl</i> -valine	50 mg. <i>d</i> -valine
	ml.	per cent	per cent	per cent
1	2.79	95	59	62
2	2.08	100	71	65
3	1.93	98	80	68
4	2.24	97	59	60
Average.....	2.26	98	67	64



a small amount of growth is observed. This growth can be almost entirely eliminated by sterilizing the *d*-leucine separately from the basal medium in

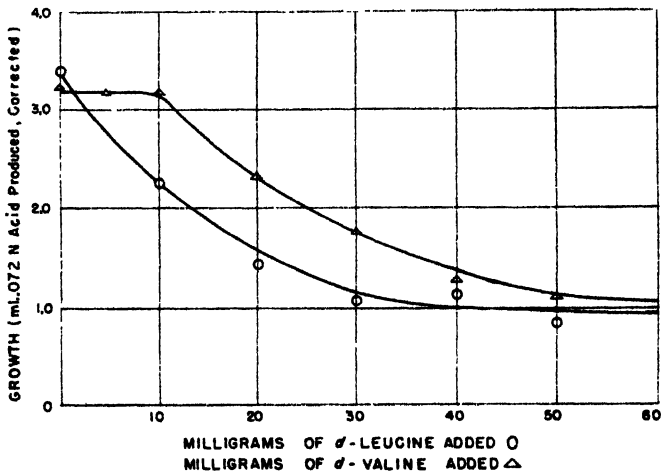


FIG. 1. Growth of *Lactobacillus arabinosus* with varying amounts of *d*-leucine and of *d*-valine added to the basal media. These curves are constructed from separate experiments.

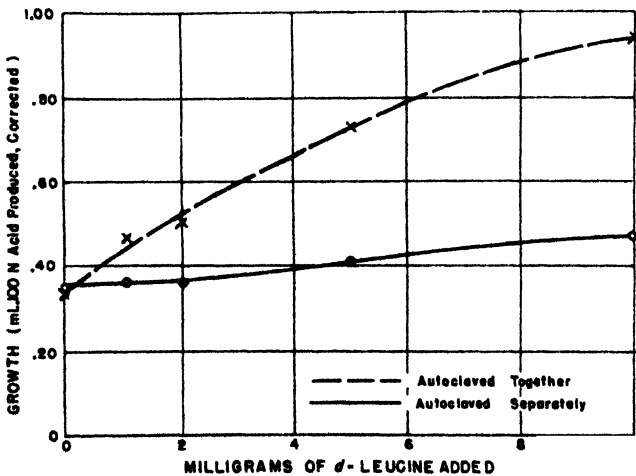


FIG. 2. Effects of autoclaving *d*-leucine together with and separately from a leucine-free medium.

each tube. The results of autoclaving various amounts of *d*-leucine and a basal leucine-free medium separately and together are presented in Fig. 2.

With amounts of added *d*-leucine of less than 1 mg., no differences were measurable. One conceivable explanation for the results observed when the *d*-leucine is autoclaved with the medium is racemization at 120° in the presence of glucose. This hypothesis cannot be tested polarimetrically, since the calculated change in rotation would be far less than the errors in measurement of rotation. The results obtained, especially when pure *d*-leucine is autoclaved separately from the medium, however, indicate agreement with the conclusion of Stokes and Gunness (8) and Kuiken *et al.* (9) that *d*-leucine is not utilized for growth by *Lactobacillus arabinosus* under usual bioassay conditions. One may contrast these inferences with that of Hegsted (10), who indicated "Probably not more than 10 per cent" of *l*-leucine activity from results with a sample of *d*-leucine reported to contain 6.7 per cent of the *l* isomer.

Because of the variation in degree of inhibition in experiments run at different times, all comparative experiments have been run on aliquots of single bacterial suspensions.

#### DISCUSSION

The results in Table I demonstrate that the inhibitory effect of mono-aminomonocarboxylic acids on the growth of *Lactobacillus arabinosus* possesses an antipodal specificity. Marked inhibition is found with the dextro configuration of amino acids possessing a relatively voluminous side chain, such as the isobutyl or isopropyl radicals in leucine and valine, but not with the methyl side chain in alanine. Such a correlation has also been found to apply to amino acids which were components of peptides subjected to the hydrolytic action of peptidase (4), carboxypeptidase (11), and papain (12). None of the *l* forms is appreciably inhibitory in either the enzymolytic or intact bacterial synthetic systems.

In both types of system, the influence of *d*-peptidase has not been adequately evaluated. The analogy is not perfect, but the same general principle of steric hindrance seems to apply. The hypothesis that the inhibitory *d*-amino acids interfere quite directly with the synthesis of protein in the bacteria seems compatible with and inferentially supported by the present state of knowledge.

It is perhaps significant that the *d*-amino acid isomers isolated from gramicidin have been those of leucine and valine. Alanine which has been obtained from this source was of the *levo* configuration only (13). Of the same group, in the present study, the *d* isomers of valine and leucine were alone inhibitory; that of alanine was not. This type of inhibition experiment also differs fundamentally from studies (10) in which *d*-leucine and *d*-valine were tested in incomplete media. The use of high levels of *d*-amino acid to study inhibition in relatively complete media as in the

present experiments is patterned after the antimetabolite to metabolite ratios which are found in antivitamin investigations (14). In the present experiments, 50 mg. of *d*-leucine represent a ratio of *d*-leucine to *l*-leucine of 200:1; a 10 mg. addition represents a 40:1 ratio.

Another correlation that may be made concerns the nutritional requirements for these amino acids. The strain of bacterium employed in this work requires leucine and valine in the *levo* configuration, whereas alanine is accessory but not essential. For such a correlation to be valid, an inhibitory *d*-amino acid would have to compete specifically with its own *l* isomer. Although some workers seem to believe in marked specificity in metabolite antagonisms (*cf.* (15)), there is experimental evidence for the contention that such antagonisms are not generally highly specific. In studying the anti-amino acid group of aminoalkylsulfonic acids, McIlwain (16) has shown, for example, that the inhibition is reversible by groups of amino acids rather than just the specific amino acid analogue of the inhibiting sulfonic acid.

The results obtained here with *d*-amino acids and the findings of Bergmann and coworkers with *d*-peptides can explain the relative non-hydrolyzability of the *Bacillus anthracis* P antigen (17) and of gramicidin and tyrocidine. The common factor would seem to be steric hindrance. As was originally suggested by Lipmann, Hotchkiss, and Dubos (18), the *d*-amino acid in peptide form may also account for some of the antibacterial activity of the peptides, especially since inhibition of bacterial growth by *d*-amino acids has now been demonstrated.

The conceivable mechanisms of inhibition of growth by *d*-leucine and *d*-valine can be grouped into two main types. Either protein synthesis is interfered with quite directly or some other type of metabolism, such as glycolysis, is slowed down. Interference with either protein synthesis or glycolysis in an intact organism can of course be expected to influence both processes. This is especially true since glycolysis is a logical source of free energy for the synthesis of the peptide linkage (19).

It is clear, however, that the bacterial processes are retarded by *d*-valine and *d*-leucine. In view of the similarity to the antipodal specificity found for proteolytic enzymes, a modification of Bergmann's picture of the inhibitory mechanism may be offered. Projections of the amino acid isomers studied are presented in the accompanying diagram. A synthesizing enzyme, according to this concept, would have reacting groups in fixed spatial arrangement. These groups would be attracted to appropriate groups of the end of the peptide chain being lengthened and simultaneously to a clockwise arrangement of the  $\alpha$ -carbon, carboxyl, and amino groups of the reacting amino acid. The enzyme, for example, is attracted to the carbon, carboxyl, and amino groups in an *l*-amino acid in a clockwise order



inhibitory. The theoretical implications of these results have been discussed.

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# ISOLATION AND PURIFICATION OF STREPTOMYCIN\*

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There has been considerable interest recently in the antibiotic agents streptothricin and streptomycin (1-11). These substances are antagonistic to Gram-negative bacteria as well as to Gram-positive. Streptomycin has a low toxicity (5) and shows promise for the treatment of infections which are resistant to penicillin (4-11). In the first reports on streptomycin and streptothricin Waksman and coworkers (1, 3) indicated that they were water-soluble organic bases. Since that time little chemical information about these substances has appeared until the recent paper of Fried and Wintersteiner (12). These authors describe certain properties of streptothricin and streptomycin, including the preparation of crystalline salts of the two bases with Reinecke acid, but give no details of the preparation of streptomycin. It is the purpose of this paper to present the methods developed in our laboratory for the isolation and purification of streptomycin.

Surface culture filtrates of *Streptomyces griseus* assaying 100 to 180 units<sup>1</sup> of streptomycin per ml. served as the starting material for this work.<sup>2</sup> Butyl alcohol fails to remove active material from an aqueous solution over a pH range of 2 to 9, and more alkaline solutions inactivate streptomycin. Streptomycin dialyzes rapidly from the culture filtrate but such a process has proved of no great practical value. A study of adsorption methods revealed that a variety of common agents removes streptomycin from an aqueous solution. The use of carbon as described by Schatz, Bugie, and Waksman (3) proved to be satisfactory, the activity being eluted from the carbon with hydrochloric acid in 95 per cent ethanol.

\* This work was supported by a generous grant from the Abbott Laboratories, Eli Lilly and Company, Parke Davis and Company, and The Upjohn Company. The authors wish to express their appreciation to these companies for the grant and also for supplies of streptomycin and streptothricin.

<sup>1</sup> The microbiological assays were made by a paper disk-agar plate method, with *Bacillus subtilis* as the test organism (in press). Thanks to the courtesy of Dr. S. A. Waksman, it was possible to adjust our standard so that the units corresponded roughly to the Waksman *Escherichia coli* dilution unit.

<sup>2</sup> Dr. H. W. Anderson and Dr. H. H. Thornberry, of the Department of Horticulture, kindly supplied us with the culture filtrates. We are happy to acknowledge our indebtedness to them.

However, anhydrous methanolic hydrogen chloride is a more convenient reagent, since the active material can be precipitated directly from the methanol solution with ether. The method of isolation as finally developed consists in clarification of the culture filtrate at pH 2 with 0.5 per cent of carbon and removal of the activity at pH 7 with 1 per cent of carbon. The carbon is washed successively with water, neutral ethanol, and neutral methanol, and the activity is then eluted by two or three extractions with 0.1 N methanolic hydrogen chloride. The neutral ethanol wash removes a large amount of material from the carbon, and a fraction of active material also is extracted. The methanolic hydrogen chloride solutions are combined and 2 to 3 volumes of ether are added, precipitating crude streptomycin chloride. The precipitate is usually a light brown amorphous powder. However, if the methanol solution contains much water, the product is a sticky gum. In either case the crude material may be purified slightly by reprecipitation from methanol with ether. The over-all recovery by this method varied from 30 to 50 per cent, and the products assayed from 150 to 300 units per mg.

The crude streptomycin chloride was subjected to several colorimetric tests in order to detect functional groups, and also in the hope of securing a rapid qualitative test which could be used in following the active material in chromatographic procedures. As was to be expected, positive tests were obtained, due to impurities. However, the Sakaguchi test for the guanidine group was positive on all active fractions and proved to be extremely useful in following purification procedures, despite the fact that certain inactive fractions also gave a positive test.

A simple method of purifying streptomycin resulted from a study of its behavior on alumina columns. Alkaline alumina removes streptomycin from neutral aqueous solutions, and elution with aqueous acid is slow and incomplete. Acid-washed alumina does not remove streptomycin from an aqueous solution but does so from aqueous methanol. This information provided the basis for a chromatographic method of purification. If a faintly acid solution of crude streptomycin chloride in 70 to 80 per cent methanol is percolated over a sulfuric acid-washed alumina column (pH 5 to 6), an inactive fraction giving a positive Sakaguchi test first appears, followed by a Sakaguchi-negative fraction. Then the Sakaguchi test rapidly rises to a peak and gradually decreases, paralleling the antibacterial activity of the fractions. A small amount of active material remains on the column and can be washed through by lowering the methanol content of the solvent. This material contains sulfate ion but no chloride. Evidently chloride ion has been replaced by sulfate from the column, and the streptomycin sulfate thus formed then passes through the column less rapidly, since the sulfate is much less soluble than the chloride in methanol.

The various fractions from the column were concentrated and lyophilized, giving white amorphous powders. The most active fractions ranged from 600 to 900 units per mg. and amounted to approximately 80 per cent of the total. Less active fractions can be purified somewhat by a second passage over the column. This technique therefore affords a simple method of obtaining potent preparations of streptomycin. Satisfactory results are obtained only if the starting material has an activity of about 200 units per mg. or higher. Less pure preparations contain substances which interfere with the development of the chromatogram.

Previous to the report of Fried and Wintersteiner (12) we had not obtained a crystalline derivative of streptomycin, and hence could not estimate the purity of our preparations. Fried and Wintersteiner isolated a crystalline reineckate which assayed 370 to 410 units per mg. and decomposed at 162–164° (corrected). They were able to convert this reineckate to the sulfate which assayed 850 units per mg. It thus appears that the more active streptomycin chloride fractions from the alumina column were approaching purity. Further, they are readily converted to a reineckate which assays 400 units per mg. and decomposes at 164–165° (corrected).

Streptomycin chloride and sulfate were obtained as white amorphous powders. The chloride is quite soluble in methanol, less soluble in ethanol, practically insoluble in butyl alcohol, acetic acid, and pyridine. The sulfate is only slightly soluble in methanol and practically insoluble in the other solvents.

Streptomycin gives a positive Sakaguchi test, and the presence of a guanidine group is also indicated by the fact that alkaline hydrolysis results in the formation of ammonia and the disappearance of the Sakaguchi test. Streptomycin also gives a positive test for the hydroxyl group (13). Negative tests are obtained in the amino nitrogen (nitrous acid and ninhydrin), Hopkins-Cole, Millon, xanthoproteic, biuret, and Pauly diazo tests. The presence of a carboxyl group is highly questionable, since streptomycin chloride, obtained by precipitation from methanolic hydrogen chloride with ether, gives an approximately neutral solution. The ultraviolet spectrum of streptomycin shows only end-absorption below 230 m $\mu$ , which makes improbable the presence of an aromatic ring or conjugated double bonds.

Streptomycin is inactivated rapidly by 0.1 N sodium hydroxide at room temperature. It is relatively stable over a pH range of 1 to 10 but is inactivated by 1 N hydrochloric acid.

Streptothricin can be isolated readily by the carbon elution method from surface culture filtrates of *Streptomyces lavendulae*. In this case the pretreatment with carbon is not necessary.



## EXPERIMENTAL

*Isolation of Streptomycin*—Surface culture filtrates of *Streptomyces griseus* grown on a peptone-meat extract medium were used. 56 liters (150 units per ml., 8.9 million units total) were acidified to pH 2.0 with concentrated hydrochloric acid, stirred for 10 minutes with 280 gm. of norit A (nuchar C-190-N is equally satisfactory), and filtered with the aid of Super-Cel. The clear filtrate (containing 7.4 million units) was adjusted to pH 7.0 to 7.5 with potassium hydroxide and stirred for 10 minutes with 560 gm. of norit A. The activity was removed completely by this treatment. The carbon was filtered and washed with water. It was transferred to a beaker and suspended in 2 liters of neutral ethanol. The ethanol was filtered and the carbon was washed in the same way with 2 liters of neutral methanol. These preliminary alcohol washes removed a considerable quantity of highly colored material and 0.9 million units of activity.

The bulk of the active material was eluted from the carbon by suspending it in two successive 3 liter portions of 0.1 N methanolic hydrogen chloride. The methanol filtrates (containing 3.4 million units) were diluted immediately<sup>3</sup> with 4 volumes of dry ether, quantitatively precipitating the activity. The crude streptomycin chloride was filtered and reprecipitated from methanol with ether. The product was washed with ether and dried *in vacuo*, giving 11.9 gm. of a friable, lightly colored, hygroscopic powder, assaying 250 units per mg. The yield of this material was 33 per cent of the original activity.

*Chromatographic Purification of Streptomycin Chloride over Alumina.* (A) *Preparation of Column*—Merck or Harshaw alumina was acidified to pH 6 with 50 per cent sulfuric acid and back-washed with distilled water until the washings were sulfate-free. At this point the supernatant liquid was at pH 6.0. The alumina was transferred to the column as a slurry, washed with a small quantity of water, and then thoroughly with 80 per cent methanol. A glass column (3.2 cm. in diameter), containing 480 ml. of packed alumina, was used for 8 to 10 gm. lots of crude streptomycin.

(B) *Procedure*—The crude streptomycin chloride (9.9 gm., 221 units per mg., 2.19 million units) was dissolved in 90 ml. of 80 per cent methanol and brought to pH 6.3 with 2.0 N lithium hydroxide in 80 per cent methanol. Brom-cresol purple was used as an outside indicator. The slight precipitate which formed was filtered and the filtrate was poured carefully into the column. The chromatogram was developed with 80 per cent methanol and the solution leaving the column was collected in 100 ml.

<sup>3</sup> The operations may be discontinued at any point prior to the treatment with acid methanol. Thereafter delay will cause considerable loss of activity.

fractions. Sakaguchi and chloride tests were made to check the location of the active material. The results of this column are summarized in Table I. As shown in Table I, inactive Sakaguchi-positive material passed through the column first. However, with the appearance of the streptomycin the intensity of the Sakaguchi test roughly paralleled the activity (Fractions 8 to 20). When the Sakaguchi color began to decrease

TABLE I  
*Fractionation of Crude Streptomycin on Alumina Column*

Fraction No.	Volume	Saka-guchi	Chlo-ride	Ac-tivity	Total units	Fraction No.	Volume	Saka-guchi	Chlo-ride	Ac-tivity	Total units
	ml			units per ml			ml			units per ml	
2	800	—	+	0		15	100	+++	+	1340	134,000
3	100	—	+	0		16	100	+++	+	1340	134,000
4	100	+	+	0		17	100	+++	+	1140	114,000
5	100	+	+	0		18	100	++	+	960	96,000
6	100	+	+	0		19	100	++	+	920	92,000
7	100	—	—	16	1,600	20	100	++	+	570	57,000
8	100	+	+	170	17,000	21	100	++	+	720	72,000
9	100	+	+	400	40,000	22	100	++	+	1060	106,000
10	100	+	+	570	57,000	23	100	+	+	570	57,000
11	100	++	+	800	80,000	24	200	+	+	800	160,000
12	100	+++	+	1140	114,000	25	200	+	—	380	76,000
13	100	+++	+	1140	114,000	26	200	+	—	400	80,000
14	100	+++	+	1110	114,000						
Total units recovered											1,715,000

TABLE II  
*Solid Fractions from Alumina Column*

Fraction No.	Weight of solid	Activity of solid	Nitrogen content
	gm	units per mc	per cent
7-11	0.45	600	11.40
12-17	1.00	900	13.47
18-23	0.65	620	13.83
24-26	0.43	520	

(Fraction 20), the 80 per cent methanol was replaced with water, which accounts for the second peak of activity. The streptomycin recovered amounted to 80 per cent of the original activity. In order to obtain solid streptomycin preparations various fractions were combined, concentrated *in vacuo* at room temperature, and dried by the lyophile process. The activity and nitrogen content of the solids are shown in Table II.

*Preparation of Streptomycin Reineckate*—A saturated aqueous solution of ammonium reineckate was added slowly to an aqueous solution of 800 units per mg. of streptomycin chloride until no further solid separated. The gelatinous precipitate readily dissolved on warming in water, and on allowing the solution to cool slowly streptomycin reineckate crystallized in the form of plates, decomposing at 164–165° (corrected). After two recrystallizations from water the product assayed 400 units per mg.

The authors wish to acknowledge the technical assistance of J. Irwin, W. Mitchell, I. Rhymer, and R. Pogrund.

#### SUMMARY

Methods are described for the isolation of streptomycin chloride from surface culture filtrates of *Streptomyces griseus* and purification of the crude material by a chromatographic process over alumina.

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# A RELATIONSHIP BETWEEN GLYCOGEN SYNTHESIS AND RESPIRATION IN LIVER SLICES

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The synthesis of glycogen from glucose by slices of rabbit liver suspended in a bicarbonate-Ringer's medium was demonstrated by Cross and Holmes (6) and by Ostern, Herbert, and Holmes (17). Unlike the synthesis *in vivo*, high concentrations of glucose must be present in the medium; otherwise glycogenolysis rather than glycogenesis occurs.

In addition we found that a high pH favored synthesis. Accordingly we studied the synthesis of glycogen by slices of rabbit liver in a bicarbonate-Ringer's medium containing 1 per cent glucose at an initial pH of 8.5. The uptake of oxygen and the respiratory quotient were also determined. A relationship was found between the synthesis of glycogen and the type of metabolism (as shown by the two respiratory factors) which is interpreted to indicate the main source of energy utilized for glycogen synthesis by the liver cell.

## EXPERIMENTAL

Young rabbits were fasted for 24 hours. Immediately following decapitation, the livers were removed and washed free of blood with saline. Slices 0.5 mm. thick and approximately 200 mg. in weight were prepared with a double razor blade cutter, weighed on a micro torsion balance, and placed with 1.5 ml. of medium in manometer vessels of a special type designed for use with bicarbonate buffer. The method<sup>1</sup> is a modification of the second method of Dickens and Simer (7). With it, the oxygen consumption, R.Q., acid production, and the change in pH are measured for the total experimental period.

The medium contained  $\text{NaHCO}_3$  0.075 M,  $\text{NaCl}$  0.045 M,  $\text{MgCl}_2$  0.002 M, and glucose 0.055 M (total osmolarity 0.300) and was equilibrated at 37° with 1 per cent  $\text{CO}_2$ -99 per cent  $\text{O}_2$ , giving an initial pH of 8.5. Calcium ions were omitted from the medium owing to insolubility of the carbonate

\* The data in this paper are taken from the thesis presented by Dana I. Crandall to the Faculty of the Graduate School of the University of Pennsylvania in 1945 in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>1</sup> Stadie, W. C., unpublished.

at pH 8.5. The medium was modified in some cases by the addition of small amounts of accessory substrates; occasionally the glucose was higher, but the total osmolarity was maintained at 0.300 by reduction of the chloride concentration.

At the end of the 2 hour experimental period, the tissue was killed by 0.4 ml. of 1 N  $\text{H}_2\text{SO}_4$  tipped into the main portion of the vessel which was then shaken for an additional hour in the bath to permit the liberation of  $\text{CO}_2$  from the bicarbonate and its subsequent absorption. This final equilibration was necessary to complete the data required for the calculation of the respiratory activity of the tissue. When the equilibration was completed, the slices were transferred to hot KOH for glycogen determination. Adequate control experiments showed that the tissues lost  $7 \pm 2$  per cent of their glycogen during this period between the termination of respiration and fixation in hot alkali.

Glycogen was determined by the method of Good, Kramer, and Somogyi (9). Changes in glycogen content were obtained by comparing the final glycogen contents of the experimental slices with values obtained from control slices which were contained in certain control manometer vessels into which the 1 N  $\text{H}_2\text{SO}_4$  was tipped at the beginning of the experimental period. These slices were added to hot KOH after a 1 hour period of shaking in the acidified medium.

Manometric determinations of pH were made at the beginning and at the end of the experimental period. The initial pH values ranged from 8.3 to 8.4 and the final pH from 7.3 to 7.7.

### Results

It is important to emphasize that the data (Table I) in each experiment were obtained with the tissue from a *single* liver.

Wide variations in glycogen formation, oxygen consumption, and r.q. are apparent in the data in Table I taken as a whole. However, when each experiment is considered separately, it is found that the variations in glycogen synthesis and oxygen uptake were interrelated in tissue slices taken from the same liver, provided they were suspended in identical media. To show this, the values for glycogen synthesis were plotted against the corresponding values for the oxygen consumption of the same slices (Fig. 1). Straight lines were drawn by inspection through the points corresponding to the data obtained with slices from the same liver suspended in the same medium.

The following conclusions were drawn: (1) An increase in oxygen consumption was accompanied by an increase in the amount of glycogen synthesized. (2) The slopes of the lines (each corresponding to a different

liver except in experiments in which two sets of slices from the same liver were studied in different media) varied from 0.02 to 0.22.

These slopes are interpreted as a measure of the extent to which an increase in oxygen consumption was accompanied by an increase in glycogen

TABLE I

*Glycogen Synthesis, Oxygen Consumption, and Respiratory Quotient of Liver Slices from Fasted Rabbits*

Bicarbonate-Ringer's medium; glucose concentration 1 per cent or greater; experimental period 2 hours at 37°; initial pH 8.3 to 8.4; final pH 7.3 to 7.7.

Experiment No.	Oxygen uptake	R Q	Glycogen increase
	<i>micromoles per gm. tissue per 2 hrs</i>		<i>mg. per gm. tissue per 2 hrs.</i>
184	173	0.72	+7.2
	154	0.71	+5.4
	191	0.79	+8.3
	198	0.71	+10.0
187	164	0.79	+11.8
	96	0.93	-3.2
189A	124	0.44	+7.4
	116	0.66	+6.8
	88	0.67	+5.0
189B*	110	0.52	+3.3
	115	0.75	+3.6
194A	139		+5.4
	206	0.67	+9.6
194B†	89	0.50	+0.4
	123	0.60	+1.3
201‡	182	0.68	+14.2
	146	0.72	+9.8
	207	0.77	+16.8
205§	199	0.81	+6.1
	226	0.76	+9.4
	242	0.68	+8.7
	174	0.70	+3.9

\* 0.01 M phlorhizin present.

† The medium consisted of Simms ox serum ultrafiltrate fortified with 0.054 M  $\text{NaHCO}_3$  and 0.050 M glucose, and equilibrated with 1 per cent  $\text{CO}_2$ -99 per cent  $\text{O}_2$ .

‡ 0.90 M glucose + 0.020 M fructose substituted for 0.055 M glucose.

§ 0.110 M fructose substituted for 0.055 M glucose.

synthesis. The slopes are designated  $\Delta$  glycogen/ $\Delta$  oxygen, and are plotted against the average R.Q. found in each set of liver slices (Fig. 2).

A direct relationship between the two variables is indicated by a correlation coefficient of  $0.92 \pm 0.06$ .

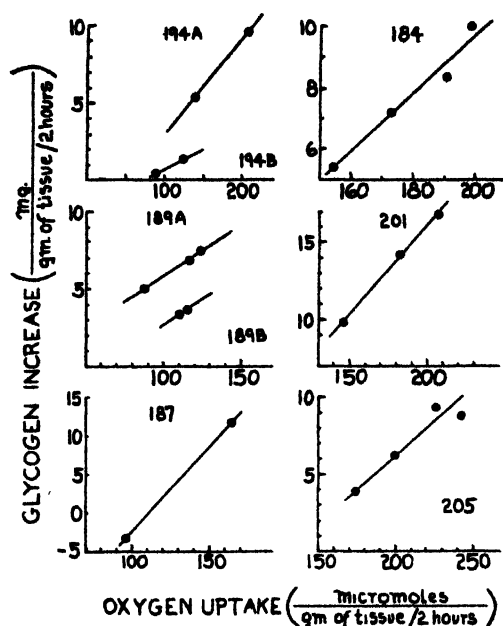


FIG. 1. Glycogen synthesis plotted against oxygen uptake in slices taken from the same liver and suspended in the same medium. Data for the experiments represented by the curves are given in Table I.

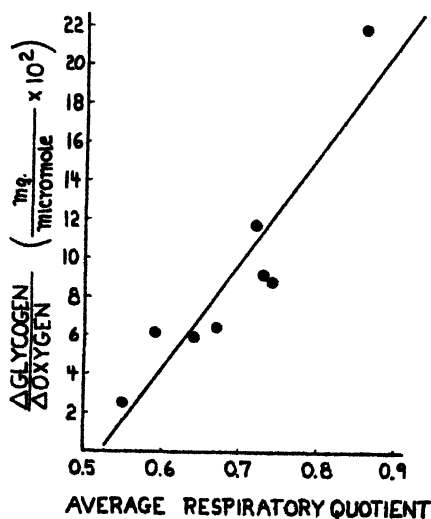


FIG. 2.  $\Delta$  glycogen/ $\Delta$  oxygen plotted against the average R.Q. Each point corresponds to a set of slices from the same liver suspended in the same medium.

From Figs. 1 and 2 it is concluded that under these experimental conditions glycogen synthesis is directly dependent on both oxygen uptake and R.Q. Further indication that a direct relationship exists between glycogen synthesis and R.Q. was obtained by comparing the average increases in glycogen for different sets of liver slices with their average R.Q. values. The advantage of this procedure is that in addition to the data cited above<sup>2</sup> it permits the use of data from eight additional experiments (not listed in Table I) in which  $\Delta$  glycogen/ $\Delta$  oxygen could not be estimated owing to

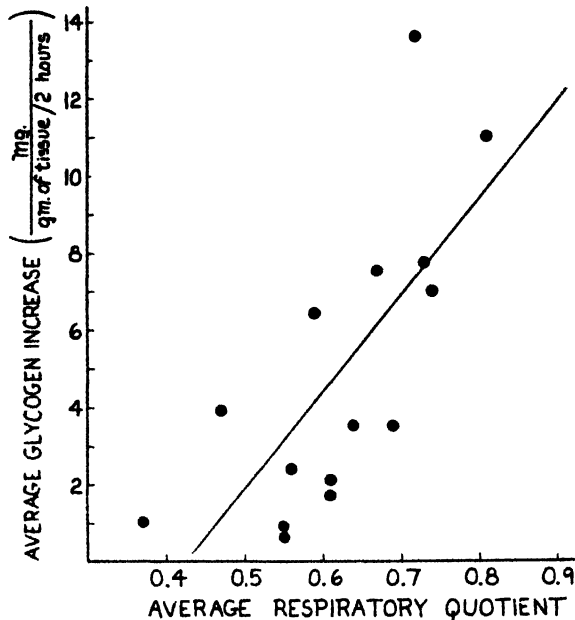


FIG. 3. The average increase in glycogen plotted against the average R.Q. Each point corresponds to a set of slices from the same liver suspended in the same medium.

the lack of significant variations in both the amounts of glycogen synthesized and the oxygen uptakes in each experiment.

A correlation coefficient of  $0.72 \pm 0.13$  between average R.Q. and average glycogen increase was calculated from the data plotted in Fig. 3.

The variations in glycogen synthesis, oxygen uptake, and R.Q. have been spontaneous rather than experimentally controlled. The variations in the oxygen uptake of different slices taken from the same liver are probably due in part to different degrees of tissue damage inherent in the slicing tech-

<sup>2</sup> Experiment 187 has been omitted from this comparison owing to the high initial glycogen levels (30 mg. per gm.) in the tissue, a factor which is known to interfere with glycogen synthesis *in vitro*.



nique and partly due to the fact that the slices were taken at random from different lobes of the liver. The variation in the average R.Q. of sets of slices taken from different livers may reflect differences in the metabolic states of the 24 hour-fasted rabbits at the time of death. The variations between the R.Q. values of individual liver slices from the same liver (see Table I) are possibly artifacts arising from errors in the measurement of  $\text{CO}_2$  production inherent in the manometric method.

#### DISCUSSION

The data presented here show that a given increase in oxygen consumption ( $\Delta$  oxygen) by the liver slices was accompanied by an increase in the amount of glycogen synthesized ( $\Delta$  glycogen) and that  $\Delta$  glycogen/ $\Delta$  oxygen was greater, the higher the R.Q. These effects are interpreted as follows:

1. The apparent dependence of  $\Delta$  glycogen on  $\Delta$  oxygen is attributed to the necessity for aerobic phosphorylation of the glucose which has been shown to be the probable first step in glycogen synthesis by the work of Cori, Colowick, and their associates on cell-free extracts of liver (3) and isolated enzyme systems (4, 19). The occurrence of this reaction *in vivo* is strongly indicated by the experiments of Kaplan and Greenberg on the distribution of radioactive phosphate in the liver phosphates of intact rats (10).

2. The direct relationship observed between glycogen synthesis and R.Q. and, in particular, between  $\Delta$  glycogen/ $\Delta$  oxygen and R.Q. is considered as evidence that the high energy phosphate required for the initial phosphorylation of the glucose arises primarily from the  $\text{CO}_2$ -producing components of the total oxidative metabolism of liver slices. Evidence that carbohydrate favors the production of this energy is found in numerous observations that glucose tolerance is highest in animals maintained on a high carbohydrate diet and lowest in those fed a high fat diet (2). Kaplan and Greenberg have shown that the level of adenosine triphosphate in the liver *in vivo* and the ability of the liver to increase this level in response to glucose administration parallel glucose tolerance in rats maintained on high carbohydrate, high protein, and high fat diets (11).

The known metabolic pathways which are capable of generating high energy phosphate are (a) the transformation of glucose to pyruvic and lactic acids by a pathway either similar to or identical with the Meyerhof glycolysis scheme which is a recognized source of high energy phosphate (15); (b) oxidations via the tricarboxylic acid cycle demonstrated for pyruvic acid in pigeon liver (8, 13, 20), and shown to involve the formation of high energy phosphate in cell-free extracts of heart (3, 16), kidney (5), and in liver *in vivo* (10, 12); (c) the oxidation of ketone bodies via the tricarboxylic acid cycle demonstrated in kidney homogenate (1). There

is no evidence, to date, that high energy phosphate arises from the conversion of fatty acids to ketone bodies, although Lehninger has shown adenosine triphosphate to be necessary for the initiation of this conversion (14).

The oxidative reactions under consideration fall into two categories with respect to R.Q. and are given in Table II.

Since ketone bodies accumulate extensively in normal liver slices (18), Reaction 4 (Table II) may be considered as a separate entity and as the main factor responsible for the low R.Q. (approximately 0.6 in the absence of substrates). An increase in R.Q. of the liver slice is an indication that an increasing percentage of the oxygen uptake is being used in  $\text{CO}_2$ -producing reactions and, conversely; a decrease in R.Q. is an indication that a greater percentage of the oxygen uptake is being utilized in a major reaction which does not yield  $\text{CO}_2$ ; namely, ketone production.

TABLE II  
*Oxidative Reactions in Liver\**

Type No	Reaction	R.Q.
1. $\text{O}_2$ consumed, $\text{CO}_2$ evolved (oxidations via tricarboxylic acid cycle)	1. Acetoacetic acid oxidation	1.0
	$\beta$ -Hydroxybutyric acid oxidation	0.9
	2. Pyruvic acid oxidation	1.2
2. $\text{O}_2$ consumed <i>without</i> $\text{CO}_2$ production	3. Pyruvic acid formation from glucose	0
	4. Ketone body " " fatty acids	0

\* Urea formation (R.Q. = -2.0) has been omitted, since it accounts for only 7 per cent of the total oxidative metabolism of liver slices in the absence of amino acids as substrates.

When the direct dependence of  $\Delta$  glycogen/ $\Delta$  oxygen on R.Q. is considered in relation to the present status of knowledge of liver metabolism, outlined above, it is felt that one of two alternative hypotheses must hold for liver tissue. Either much more high energy phosphate arises per unit of oxygen consumed by the  $\text{CO}_2$ -producing component of the total oxidative metabolism than by the conversion of fatty acids to ketone bodies, or, in the event that the conversion of fatty acids to ketones gives rise to as much (or more) high energy phosphate per unit of oxygen consumed as the  $\text{CO}_2$ -producing oxidations do (the tricarboxylic acid cycle), this energy is unavailable for glycogen synthesis.

The author wishes to express his sincere appreciation to Dr. William C. Stadie for his kind interest and valuable advice during the course of this work.

## SUMMARY

Direct relationships between glycogen synthesis and oxygen consumption and between glycogen synthesis and respiratory quotient were observed in liver slices, from fasted rabbits, suspended in a bicarbonate-Ringer's medium containing glucose.

The data are interpreted as evidence that the energy needed for the synthesis of glycogen from glucose in liver tissue arises primarily from oxidations which give rise to  $\text{CO}_2$ .

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# CELL STRUCTURE AND THE PROBLEM OF BLOOD COAGULATION\*

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The cardinal function of tissue cells in initiating the process of blood coagulation has long been known. The recognition of the presence in cells of a water-soluble clotting activator (1-4), regarded as a general constituent of the protoplasm (5), has led many workers to assign to it a pivotal rôle within the coagulation phenomenon.

Studies, carried out in this laboratory during the past few years, have drawn attention to the occurrence in lung tissue of a lipoprotein of very high particle weight, the thromboplastic protein, which proved to be an extremely potent activator of prothrombin. A summary of the properties of this substance will be found in a recent review (6). Whether the thromboplastic activity exhibited by crude lung extracts was all ascribable to the thromboplastic protein or whether other cell fractions likewise had similar properties remained, however, undetermined. The present study will provide a comparative survey of the thromboplastic activity of representative cellular fractions isolated from lung tissue.

The difficulty of correlating what the cytologist observes in a cell with what the chemist isolates from it is obvious and requires no comment. Apart from the tenuous support provided by color reactions and optical observations, the evidence is meager indeed. (A discussion of some of these aspects will be found in recent monographs (7, 8).) The fractions examined in the present study for thromboplastic activity comprise, in addition to the cell nuclei, all non-dialyzable substances that can be extracted with physiological saline from lung tissue. A variety of cell species must, of course, have contributed to these fractions which include (1) the thromboplastic protein, a macromolecular lipoprotein containing some pentose nucleic acid (9, 10), considered, by present views, to be of cytoplasmic origin;<sup>1</sup> (2) coarse tissue particles sedimenting at between 1900 and 5000 *g*, consisting of material of unknown origin together with varying

\* This work has been supported by a grant from the John and Mary R. Markle Foundation. This is Paper XIX of a series of studies on the chemistry of blood coagulation.

<sup>1</sup> The direct observation of submicroscopic cytoplasmic particles was recently made possible by electron microscopy (11).

amounts of the thromboplastic protein that became aggregated in the course of the manipulations; (3) the proteins (consisting of four electrophoretic components) extractable from the tissue with physiological saline and not sedimentable by high speed centrifugation which form the bulk of the extract and probably represent a mixture of both intercellular and intracellular substances; and, finally, (4) the cell nuclei.

A satisfactory efficiency of fractionation is attested to by the distribution of acetal phosphatides and of desoxypentose nucleic acid in the various fractions examined. The demarcation lines were quite sharp: acetal phosphatides were found only in the heavy tissue particles and in the thromboplastic protein, desoxypentose nucleic acid only in the nuclei. Even more fortunate for the purposes of the present study was the finding that the thromboplastic activity was practically all concentrated in one cell fraction; *viz.*, the thromboplastic protein sedimenting from lung extracts at 31,000 *g*. The coarser aggregates sedimenting at an intermediate centrifugal speed exhibited some activity, but this was probably due to an admixture of some compacted thromboplastic protein, since in previous experiments (10) comparable fractions were encountered that were almost devoid of activity. The other fractions examined, *viz.* the nuclei and the non-sedimentable tissue proteins, contained only minute traces of thromboplastic activity.

It is too early to attempt a delineation of the relationship between the physiology of blood clotting and what might be called its cytology, although the discovery that the thromboplastic activity is localized in one definite macromolecular fraction of the cytoplasm may, at a later date, prove of importance. Size, in compounds having biological activity, is probably not an accident. It is significant that the only cell fraction endowed with thromboplastic activity has the enormous particle weight of 170 million (9), a fact that will, among other things, influence the rate at which the active substance diffuses, once the cell wall is ruptured. The question may be raised whether, in view of the ability of tissue particles to adsorb enzymes, the thromboplastic agent should be considered as forming an integral part of the large structure. It is not yet possible to give a definitive answer, but it should be remembered that almost no activity could be detected in the supernatants following the sedimentation of the thromboplastic protein and that under conditions in which a considerable amount of phosphatase could be detached from the macromolecular complex the thromboplastic activity remained combined with it (10).

Heparin is known to be present in lung tissue in a high concentration (12). None of the fractions examined in this study, however, gave the metachromatic reaction with toluidine blue (13). When the protein residue resulting from the treatment of the thromboplastic protein with alcohol-

ether (10) was heated to 70° with 0.5 N potassium hydroxide, the neutralized mixture gave a weak metachromatic reaction. It is not impossible, therefore, that heparin was present in the protein complex in such a combination as to make it unavailable for the color test without previous drastic treatment.

The method for the comparative assay of thromboplastic activity, discussed in detail in the experimental part, is based on the use of normal human plasma deprived, by high speed centrifugation, of a large proportion of the thromboplastic factor (compare (14)).

## EXPERIMENTAL

### *Determination of Thromboplastic Activity*

*Effect of High Speed Centrifugation on Clotting Time of Human Plasma*—Normal human blood, collected before breakfast, was mixed immediately

TABLE I  
*Effect of Centrifugation on Plasma Clotting Time*

Experiment No.	Duration of centrifugation	Centrifugal force	Clotting time*	
			Plasma 1	Plasma 2
	<i>min.</i>	<i>g</i>	<i>sec.</i>	<i>sec.</i>
1	3	260	130	125
2	20	1,900	220-290	217-250
3	150	31,000	365-690	340-690

\* The figures reported for Experiments 2 and 3 indicate the span between the onset of coagulation and the formation of a coherent clot.

with one-ninth its volume of a 0.1 M sodium oxalate solution. Plasma samples were removed following the centrifugation of the mixture (in Lusteroid cups) at 1500 R.P.M. (260 *g*) for 3 minutes and at 4000 R.P.M. (1900 *g*) for 20 minutes in an angle centrifuge. The clear plasma was drawn off carefully and subjected to a centrifugation at 20,000 R.P.M. (31,000 *g*) for 150 minutes in a refrigerated International centrifuge equipped with a multispeed attachment, whereupon a minute reddish brown pellet separated at the bottom of the Lusteroid tubes. The clotting times, observed at 37° following the addition of 0.2 cc. of a 0.01 M calcium nitrate solution (containing 0.42 per cent of sodium chloride) to 0.1 cc. of the plasma samples, are recorded in Table I. It may be mentioned that the addition of the suspended high speed sediment to the supernatant plasma brought about a very considerable shortening of the clotting time. This will be discussed in detail on a later occasion. What is of importance here is that the plasma obtained by centrifugation at a high speed (Experiment 3 in Table I), which had been

deprived of an appreciable portion of the thromboplastic factor, could be used for the assay of thromboplastic activity.

*Assay*—The determinations were carried out in small test-tubes (13 × 100 mm.) at 37°. To a mixture of 0.1 cc. of oxalated plasma, obtained by high speed centrifugation, and 0.1 cc. of a suspension of the substance to be tested in physiological saline, 0.2 cc. of a 0.01 M calcium nitrate solution (containing 0.42 per cent of sodium chloride) was added. While in high concentrations of the thromboplastic protein no time lag between the start and the completion of coagulation could be observed, there was a noticeable span in the presence of very small amounts of the active substance, and care was taken to record the times both at which a clot became perceptible and at which it became coherent.

The main advantage of the method consists in the ease with which both highly and slightly active fractions can be tested over a wide range (usually a decimal series) of dilutions. The accuracy of the results suffers, especially in low concentrations of the active substance, from the usual limitations of determinations of this kind; *viz.*, dilution errors and the difficulty with which the end-points are ascertained. Nevertheless, with the same plasma sample representative data could be obtained which compared favorably with those given by the previously used assay method in which rooster plasma was employed (15). The plasma (stored in the refrigerator) was never used for more than 2 days after its preparation.

*Presentation of Assay Results*—For purposes of comparison, the results are plotted as the logarithms of the concentration (in micrograms) of the active substance against  $1000/t$  where  $t$  is the clotting time (in seconds). In low concentrations of the activator, *i.e.* with clotting times of more than 200 seconds, the first appearance of a coagulum was used as a more reliable measure than the formation of a coherent clot.

Because of limited experience, it cannot yet be decided whether the values obtained by this method may be used for absolute potency determinations. It will perhaps be possible to overcome the fluctuations observed with different plasma samples (Fig. 1) by employing a standard preparation of the thromboplastic protein alongside the unknown specimens. The results of four independent assays of Fraction 2 (Table II) with three different plasma samples are shown in Fig. 1. The thromboplastic activities of the different cell fractions discussed in this paper are compared in Fig. 2, the same plasma sample being used as the substrate throughout. It will be seen that with high concentrations of the active substance, *i.e.* presumably in the region of excess of the thromboplastic protein, a linear relationship is observed, while in low concentrations (or with only slightly active fractions) a change in slope appears to take place.

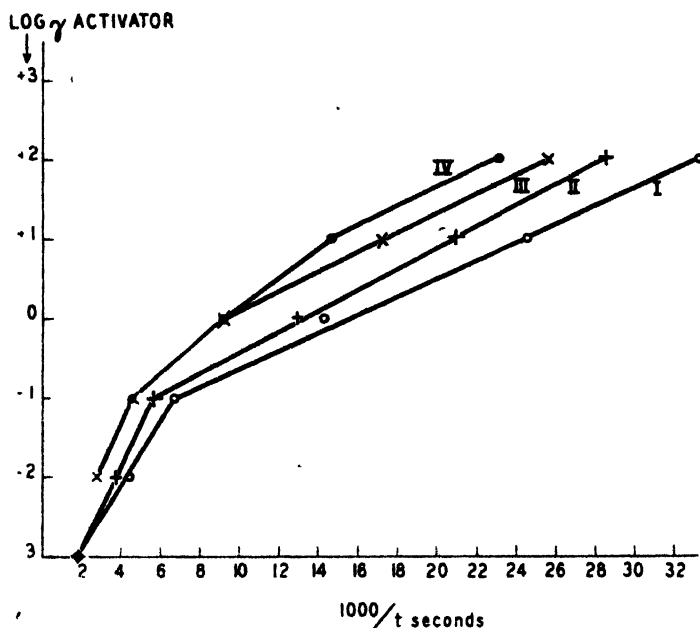


FIG. 1. Coagulation of normal human plasma (obtained by high speed centrifugation) by the thromboplastic protein (Fraction 2). In the expression  $1000/t$ , plotted as the abscissa,  $t$  corresponds to the clotting time in seconds. Curves I, II, and III were obtained with three different 1 day-old samples; for Curve IV the same sample as for Curve III was used, but 2 days after the removal of the blood.

TABLE II  
*Composition of Cell Fractions from Beef Lungs*

Fraction No.	Designation	Yield per kilo tissue	N	P	Acetal phosphatides (Feulgen and Grünberg (16))	Desoxy-ribose nucleic acid (Dische (17))
		gm.	per cent	per cent		
1	Coarse lipoprotein aggregates	0.73	7.0	2.0	+	—
2	Thromboplastic protein	1.12	8.0	1.7	+	—
3a	Soluble proteins	0.21	14.6	0.8	—	—
3b	" "	10.27	15.0	0.2	—	—
4	Cell nuclei	1.96	13.8	1.5	—	+

*Distribution of Thromboplastic Protein in Different Cell Fractions of Beef Lungs*

The following experiments were all performed on the same specimen of fresh beef lungs. The separation procedure will be made clear by the



accompanying flow sheet. It followed, with respect to the thromboplastic protein, the methods described in previous publications (9, 10). A refrigerated International centrifuge equipped with a multispeed attachment was used throughout for the separations undertaken at high centrifugal speeds. All operations were carried out at 4°. Analytical and other data concerning the various fractions are assembled in Table II. A few additional details follow.

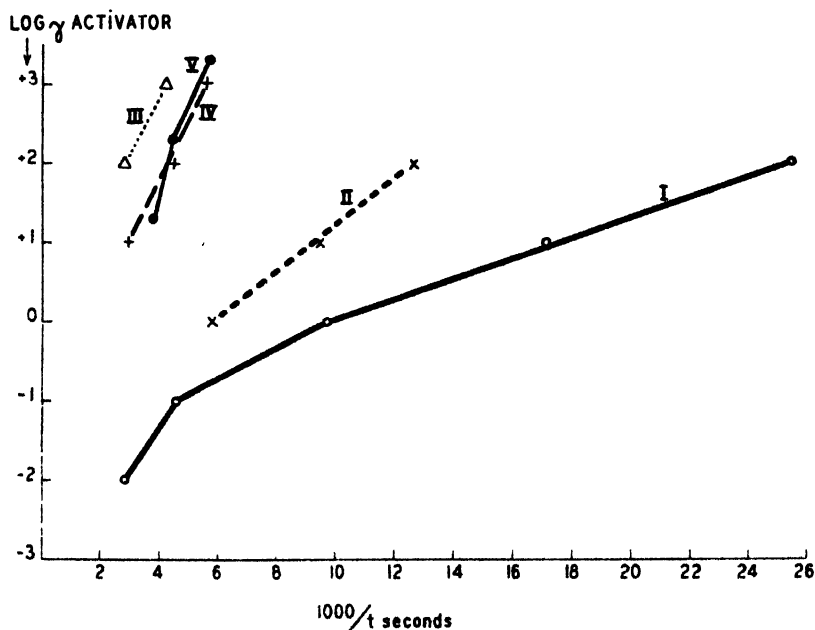


FIG. 2. Coagulation of normal human plasma (obtained by high speed centrifugation) by cellular fractions from beef lungs. Curve I, Fraction 2; Curve II, Fraction 1; Curve III, Fraction 3a; Curve IV, Fraction 3b; Curve V, Fraction 4 (compare Table II).

*Coarse Lipoprotein Aggregates (Fraction 1, Table II)*—The borate suspension of the washed thromboplastic protein was subjected to a centrifugation at 8000 R.P.M. (5000 *g*) for 30 minutes. The sediment which consisted of a portion of the thromboplastic fraction that had become aggregated during the preparation, together with some coarser material, was suspended in borate buffer of pH 8.4 and the mixture centrifuged at 4000 R.P.M. (1900 *g*). The supernatant and the washings obtained in the same manner were dialyzed for 48 hours against running tap water and for 117 hours against ice-cold distilled water. The frozen suspension was concentrated to dryness *in vacuo*, when Fraction 1, 720 mg. of a white fluff, was

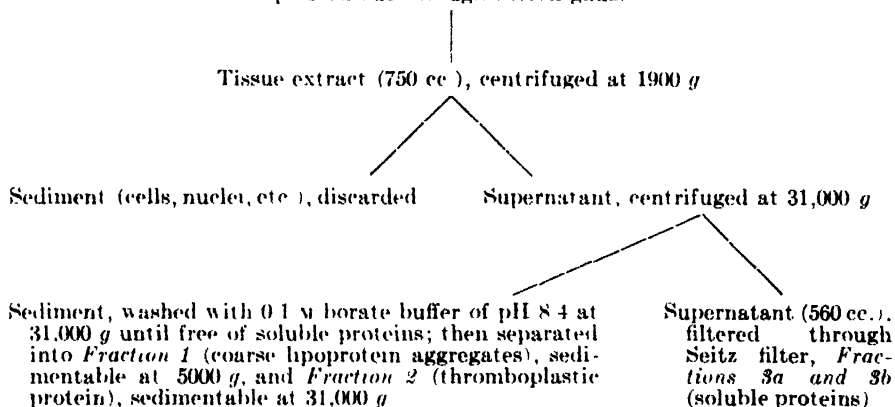
obtained. The thromboplastic activity of this material is represented in Fig. 2.

*Thromboplastic Protein (Fraction 2, Table II)*—This material was isolated in the usual manner (9, 10): *Fraction 2*, 1.10 gm. of an almost white voluminous felt. The thromboplastic activity of this substance is shown in Fig. 2.

*Soluble Proteins (Fractions 3a and 3b, Table II)*—A portion (400 cc.) of the bright red supernatant obtained by the centrifugation of the tissue extract at 31,000 *g* (see the flow sheet) was filtered through a Seitz filter. The clear filtrate was dialyzed for 66 hours against running tap water and for 24 hours against ice-cold distilled water. During the dialysis a small amount of flocculent precipitate separated.

#### Separation Procedure

Ground beef lungs (985 gm.), extracted with 1000 cc. of physiological saline and pressed out through cotton gauze



For electrophoresis, a sample of the electrolyte-free mixture was dialyzed against 0.1 M borate buffer of pH 8.4, when a clear solution resulted. The electrophoretic analysis<sup>2</sup> revealed the presence of four components, of which one preponderated, with the following mobilities (descending boundaries): I, -1.4 (minute component); II, -3.7 (main component); III, -5.3 (minute component); IV, -9.0 (small component)  $\times 10^{-5}$  sq. cm. per volt per second.

The remaining mixture was centrifuged and the sediment washed repeatedly with distilled water and dried in the frozen state *in vacuo*: *Fraction 3a*, 140 mg. of pink flakes. The water-soluble portion was similarly recovered: *Fraction 3b*, 6.5 gm. of voluminous pink flakes. Both fractions exhibited only a trace of thromboplastic activity (compare Fig. 2).

<sup>2</sup> We are greatly indebted to Dr. D. H. Moore for this determination.

*Cell Nuclei (Fraction 4, Table II)*—The preparation of cell nuclei from lung tissue proved much more difficult than that of liver nuclei. Out of several runs at pH 5.9 only one was successful. No nuclei could be prepared at pH 3.7.

The method of isolation was essentially similar to that described by Dounce (18) for liver tissue and will, therefore, be presented only briefly. Small washed pieces of fresh beef lungs (100 gm.) were frozen and gradually added to a mixture of 250 gm. of crushed ice, 250 cc. of distilled water, and 1 cc. of *m* citric acid that was rotated in a high speed mixer. When the tissue and ice had disintegrated, which took about 25 minutes, the mixture (pH 5.9) was passed through three layers, and the filtrate through five layers, of cotton gauze. The sediment, obtained by centrifugation at 4000 R.P.M. (1900 *g*) for 20 minutes,<sup>3</sup> was twice washed with 350 cc. portions of distilled water by centrifugation at the same speed for 10 and 5 minutes respectively, and then three times with 200 cc. portions at 1500 R.P.M. (260 *g*) for 3 minutes. At this stage, the washings were no more turbid and contained practically no protein. The sediment, finely suspended in 100 cc. of distilled water, was permitted to settle in a graduated cylinder at 4° for several hours; the suspension was siphoned off with the exception of the undermost 5 cc. which were again dispersed in a total water volume of 100 cc. and submitted to the same settling process for 1 hour. The dense bottom portion (5 cc.) was discarded and the united supernatants were centrifuged. The sediment was suspended in a small volume of water and, after removal of samples for microscopic examination, dried in the frozen state *in vacuo*. The nuclei, *Fraction 4*, weighed 196 mg. and formed cork-colored flakes.

The microscopic examination of unstained preparations showed beautiful intact nuclei; observations on stained specimens (Zenker's fluid, hematoxylin-eosin) revealed the presence of about 80 per cent of pure nuclei, the rest consisting of nuclei contaminated with some cytoplasmic material.<sup>4</sup> In unsuccessful preparations, gross contamination, mostly with elastic fibers, was observed at this stage. The reason for these frequent failures is not yet clear.

The nuclear material was, for the determination of its thromboplastic activity, intimately suspended in physiological saline by means of a small Pyrex glass grinder. The potency of this fraction was very low (Fig. 2).

The technical assistance rendered by Miss Helen Fabricant is gratefully acknowledged.

<sup>3</sup> A preparation of the thromboplastic protein, somewhat less active than *Fraction 2*, could be obtained from the supernatant.

<sup>4</sup> We are very grateful to Dr. D. Cowen for some of the microscopic examinations.

## SUMMARY

A study of the distribution of thromboplastic activity in representative cellular fractions from beef lungs revealed that practically all the activity was confined to one fraction, the thromboplastic protein, sedimentable from lung extracts at 31,000 *g*. The other fractions examined (coarse tissue particles, non-sedimentable tissue proteins, cell nuclei) had practically no activity. A method for the assay of thromboplastic activity with normal human plasma, deprived of most of the thromboplastic agent by high speed centrifugation, is described.

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# THE DETERMINATION OF THIAMINE IN URINE BY MEANS OF THE THIOCHROME TECHNIQUE\*

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Many workers have observed that urine contains substances which interfere with the estimation of thiamine by the thiochrome technique (1, 3, 6, 7, 9). Some of the substances are removed by the permittit treatment in the ordinary procedure, but others are carried through in the eluate. Compensation for these compounds is often attempted by the use of a urine blank in which only the ferricyanide is omitted, the assumption being that the ferricyanide has no effect on the non-thiamine fluorescent substances in urine. It is readily shown that this assumption is not valid. The unsatisfactory state of current procedures is most clearly seen in the frequent finding, in urines from persons on low thiamine intakes, of markedly negative thiamine values; i.e., where the blank is much greater than the thiochrome value.

We have been able to show that the amount of non-thiamine fluorescent materials in urine is often much larger than commonly suspected, that the non-thiamine fluorescence is greatly affected by ferricyanide, and that these interfering substances may be eliminated, at least in large part, by suitable adjustments in pH at the final extraction stage. The procedure developed as a result of systematic investigation has been carefully studied in terms of the elimination of negative values, recovery of added thiamine, prediction of the values for mixtures of high and low thiamine urines, and in agreement of duplicates.

*Influence of pH on Extraction of Thiochrome*—A standard containing 10  $\gamma$  of thiamine in 100 ml. of 25 per cent KCl in 0.1 N HCl was prepared. 5 ml. portions of this standard solution were treated with 3 ml. of 15 per cent NaOH and 0.1 ml. of 1 per cent potassium ferricyanide to convert the thiamine to thiochrome. These thiochrome solutions were then brought to different acidities by the addition of a mixture of equal volumes of concentrated HCl and 85 per cent  $H_3PO_4$ , and the pH was measured with the glass electrode. These solutions were each extracted with 15

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ml. of isobutanol, the aqueous phase was removed, and the isobutanol solutions were dried with 2.5 gm. of anhydrous  $\text{Na}_2\text{SO}_4$ . Fluorescence was measured in the Coleman photofluorometer (model 12). Fig. 1 shows that the extraction of thiochrome is independent of acidity between pH 8 and 10 but is diminished below pH 7.

The non-thiamine fluorescent materials in urine behave very differently. A urine sample which exhibited a high blank value in the ordinary thiochrome procedure was acidified to pH 4.5. 5 ml. portions of this urine

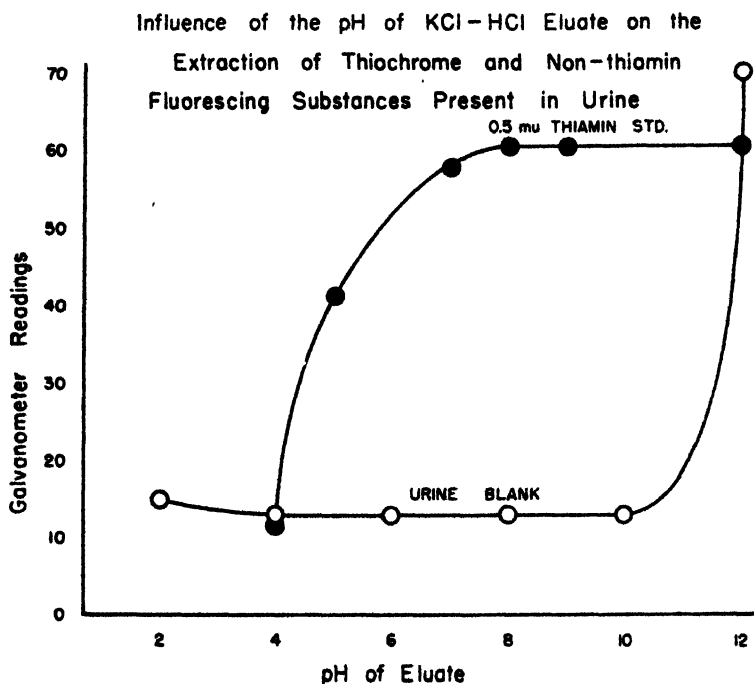


FIG. 1

were poured into each of six standard permutit columns. Each column was washed with water and then eluted with 15 ml. of boiling KCl-HCl solution. 5 ml. of each eluate were treated with 3 ml. of 15 per cent NaOH solution and then adjusted to different acidities with  $\text{HCl-H}_3\text{PO}_4$  as in the standards, and the remaining procedure carried out as before. The results are shown in Fig. 1. These findings indicate that when extraction is made from a solution at pH 8 to 10 the non-thiamine fluorescent substances in urine are largely eliminated, but that the extraction of thiochrome is unimpaired.

### *Method*

The main reagents are those customarily used for the thiochrome determination (8). The urine is collected in a bottle containing 5 ml. of glacial acetic acid and 5 ml. of toluene for a 24 hour sample. Under ordinary circumstances a pH of 4 to 5 will result, but this should always be checked before the analysis is made. The towers for the permutit are made with a capillary 1 cm. long, which has an internal diameter of 0.5 mm. With this size of capillary no cotton or glass wool is required at the bottom of the tower to prevent the permutit from plugging the tube. Occasionally a particle does plug the hole, but it can be removed by inserting a 32 gage wire. This type of tower requires no suction, for the liquids emerge at approximately the proper rate.

10 ml. of 3 per cent acetic acid are added to the tower by means of a pipette in such a way that the sides of the reservoir are not wet. There should be enough acid to fill the tower and a small part of the reservoir. 1 gm. of permutit is then added from a calibrated vial. Any permutit which clings to the sides of the reservoir is washed down with more acetic acid. This procedure obviates the formation of bubbles which ordinarily occurs when the dry permutit is added to the tower first.

After the acetic acid has drained from the tower, 5 ml. of the acidified urine are put through,<sup>1</sup> followed by washing with 3 successive 10 ml. volumes of water. When the wash water has drained, the thiamine is eluted with 15 ml. of boiling KCl-HCl solution. This is permitted to drain from the tower. On successive trials, the volume of the eluate has been  $14.75 \pm 0.25$  ml. The eluate is mixed by shaking. 5 ml. of the eluate are then added to a conical shaped tube,<sup>2</sup> containing 3 ml. of 15 per cent NaOH and 0.1 ml. of 1 per cent potassium ferrieyanide. The tube is vigorously shaken for a few seconds. From 0.50 to 0.55 ml. of a mixture of equal parts by volume of concentrated HCl and 85 per cent  $H_3PO_4$  is added to the alkaline eluate; the end-point (pH 8 to 9.5) is sufficiently indicated by the formation of a white precipitate. 15 ml. of redistilled isobutanol are added (most conveniently with a syringe pipette (2)) and extraction is accomplished by agitation produced by a stream of air for 30 seconds. After the tube has stood for about 5 minutes, the aqueous phase can be cleanly removed by suction. The isobutanol phase is dried with 1.5 gm. of anhydrous  $Na_2SO_4$  and read in a photofluorometer. Thiamine solutions of known concentration are put through the same pro-

<sup>1</sup> If the concentration of thiamine is greater than 1.5  $\gamma$  per 5 ml., a volume of urine should be used which contains this amount or less. Even when the thiamine concentration is very low, the volume used for analysis should never be greater than 5 ml.

<sup>2</sup> This tube is about 16 cm. long and 2.8 cm. wide, internal diameter, with the lower 3 cm. of the tube tapered.



cedure for calibration of the standard curve. The urine blanks are also treated in precisely the same way, except for the omission of the ferricyanide.

*Recovery Experiments*—Recovery experiments were made on a variety of urines by both the present and the ordinary thiochrome procedures (Table I). None of the added thiamine was recovered by the ordinary procedures from urines which had negative values before such addition, although the negativity, that is the difference between the urine blanks and the thiochrome reading, was considerably reduced. With the present procedure recoveries of from 91 to 103 per cent were obtained in these same urines, and equal or better results were obtained with urines containing initially high thiamine concentrations. The recovery of thiamine

TABLE I

*Recovery Experiments with Pure Thiamine Added to Urine*

The recovery experiments were made both with the old and the present methods. The first four urine samples showed a higher galvanometer reading for the urine blank than for the thiochrome reading when analyzed by the old procedure.

The values are in micrograms per 1.67 ml. of urine.

Ordinary method					Present method			
Sample	Thiamine content	Thiamine added	Recovered thiamine		Thiamine content	Thiamine added	Recovered thiamine	
				per cent				per cent
P8-30...	0	0.167	0.00	0.0	0.088	0.167	0.152	91.0
S8-30	0	0.167	0.00	0.0	0.050	0.167	0.172	103.0
W9-2	0	0.167	0.00	0.0	0.026	0.167	0.168	100.6
J9-2..	0	0.167	0.00	0.0	0.056	0.167	0.160	95.8
H10-13...	0.057	0.200	0.179	89.5	0.096	0.200	0.197	98.5

added to so called low blank urines is reasonably good by both procedures, but the present method consistently gives a somewhat better recovery.

Another type of recovery experiment was made by mixing equal parts of a high blank urine and a low blank urine (Table II). Each of the separate urines was analyzed by both procedures. When the combined sample was analyzed by the ordinary procedure, only 50 per cent of the theoretical value was secured; this figure is based on the separate values of the two urines as estimated by the same method. In contrast the present procedure gave a recovery of 103 per cent.

It appears that urine, particularly that which has a high blank in the ordinary procedure, contains a substance or substances which are fluorescent, which are extracted by isobutanol at high alkalinity, and which show much less fluorescence when treated with ferricyanide. Recoveries may appear reasonable when the urine contains much thiamine and little

of the interfering material, but the recoveries are poor with the ordinary procedure in the reverse situation. This is particularly important since little thiamine and much interfering material in urine frequently occur when the thiamine nutrition is very poor; that is, precisely when the urine values are most interesting. The difference in blanks and thiamine

TABLE II

*Thiamine Values on High Blank and Low Blank Urines When Mixed in Equal Proportions*

The theoretical values are the averages of the thiamine contents determined on the individual samples. The actual values were determined on the mixed urine sample. All values are in micrograms per 1.67 ml. of urine.

Sample	Ordinary method	Present method
Frank 8-31. . . . .	0	0.143
Sel 9-6 . . . . .	0.527	0.563
Mixture of equal parts of above		
Theoretical . . . . .	0.263	0.353
Actual . . . . .	0.136	0.365
$C_c$	49.8	103.4

TABLE III

*Comparison of Galvanometer Readings and Indicated Thiamine Values for Urine Samples with Ordinary and Present Procedures*

The reagent blank in each case represents 5 ml. of distilled water carried through the entire procedure. The urine blanks are the values for the urine eluates treated with the NaOH solution, whereas the unknown values are for the urine eluates treated with the alkaline ferriyanide. All readings are made on a Coleman photo-fluorometer set so that a 0.5  $\gamma$  standard reads 63 by the present method.

Method	Galvanometer readings			Thiamine		
	Reagent blank	Urine blank	Thiochrome	Urine blank	Thiochrome	Difference
Ordinary. . . . .	11.0	21.0	27.0	$\gamma$ 0.098	$\gamma$ 0.155	$\gamma$ 0.057
Present . . . . .	12.8	15.4	25.0	$\gamma$ 0.027	$\gamma$ 0.123	$\gamma$ 0.096

values by the ordinary and the present procedure is illustrated in Table III.

*Experiments with Diets and with Unadsorbed Urines*—An attempt was made to use the present procedure with urines that had not been adsorbed on permutit in the hope that, at the proper pH, the non-thiochrome fluorescent substances would not be extracted with isobutanol. This, however, was not the case. Without adsorption the blank values of ordinary urines were as high as or higher than the thiochrome readings.

When the present procedure is used with food materials, the thiamine values tend to be the same as those secured by the ordinary procedure (Table IV). In the case of Sanka coffee the blank value by both methods is high, an instance where non-thiamine fluorescent substances are extracted by isobutanol to the same extent over a wide range of pH. Most other foods produce blank values which are very low with either procedure.

TABLE IV

*Comparison of Thiamine Values on Foods by Ordinary and Present Procedures*

All values are in micrograms per 100 gm. of food.

Food	Thiamine content	
	Ordinary method	Present method
Beef and gravy	14.8	15.4
Meat and vegetable stew	26.0	26.0
Mixed diet	20.2	20.0
Cocoa	89.5	90.0

TABLE V

*Relation of Thiamine Blank to  $F_2$*

$F_2$  excretion determined by the method of Najjar (5). The thiamine blank was determined by the ordinary thiochrome technique in which the ferricyanide was omitted and no pH adjustment was made.  $F_2$  is expressed as fluorescence equivalent to mg. of quinine sulfate. The thiamine blank is expressed as mg. of thiamine per day.

Subject	Date	$F_2$ excretion per day	Thiamine blank	Thiamine blank* $F_2$ excretion
Jo . . .	Aug. 21	0.553	0.204	0.369
Ja . . .	Sept. 2	0.509	0.175	0.344
T. . . . .	" 2	0.280	0.115	0.410
P . . . .	Aug. 30	0.726	0.870	1.20
S . . . .	" 30	0.549	0.246	0.448
W . . . .	Sept. 2	0.229	0.095	0.042
G . . . .	" 2	0.219	0.079	0.036

\* Ratio of thiamine blank to  $F_2$  content of urine.

*Comparison with Procedure of Najjar and Ketron*--Najjar and Ketron (6) stated that the blank fluorescence in urine is due to the niacin derivative  $F_2$  and showed that ferricyanide reduces the fluorescence of  $F_2$  by conversion to a pyridone with only 21 per cent of the fluorescence of the original pyridine derivative,  $F_2$ . On this basis they proposed a formula for correcting the thiochrome reading according to the  $F_2$  content of the urine. The limited validity of this calculation is indicated by the fact that the ratio

between the thiamine blank and the  $F_2$ , as determined by the method of Najjar (5), is inconstant and is especially unreliable in high blank urines. In one series of seven men the ratio between thiamine blank and  $F_2$  in the urine varied from 0.036 to 1.20 (Table V).

Najjar and others have emphasized that the excretion of  $F_2$  in the urine is directly related to the niacin intake. We have found that a decrease in the niacin intake from 32 to 12 mg. per day, *i.e.* a change of 167 per cent, does not cause any considerable change in the thiamine blank as obtained by the ordinary procedure. Apart from the fact that  $F_2$  is not the principal cause of the thiamine blank in many urines, it is of interest that the method advocated by the Johns Hopkins group systematically yields values higher than those obtained by the present procedure. The difference is particularly impressive on a percentage basis in urines of low thiamine content (Table VI).

TABLE VI

*Comparison of Thiamine Values by Present Method with That of Najjar and Ketron (1944)*

Subject	Date	Thiamine content of urine per 100 ml.	
		Najjar and Ketron	Present method
		$\gamma$	$\gamma$
A	Oct. 30	13.02	9.3
B	Dec. 20	20.5	15.4
P	Aug. 30	12.12	5.28
S	" 30	12.2	3.00
W	Sept. 2	4.50	1.56

*Influence of Volumes Used in Analysis*—The starting volume of urine used in the analysis has a profound influence on the thiamine value secured. In general, the larger the starting volume, the smaller is the final value. At present we find it advisable to use no more than 5 ml. of urine, to elute with 15 ml. of KCl-HCl, and extract 5 ml. of this eluate with 15 ml. of isobutanol. By using the maximum sensitivity of the photofluorometer, even the low thiamine urines can be determined with a fair degree of accuracy.

*Order of Addition of Reagents*—If the eluate from an adsorbed standard thiamine solution is added to the alkaline ferricyanide, the galvanometer reading for the final isobutanol extract is higher than when the order of addition is reversed. For a 0.5  $\gamma$  standard the galvanometer readings average 65 and 63 respectively. This effect of the order of addition holds true only for pure thiamine solutions; no difference is observed in the case of urine or food eluates.

*Reliability of Present Procedure*—The simplest estimation of reliability is obtained by the statistical analysis of the agreement between duplicates. Abundant material is available for this purpose due to the fact that all urine thiamine estimations in this laboratory are routinely made in duplicate. One of the routine note-books was opened at random and the values for thirty-three consecutive pairs of duplicates were examined. In a series of 66 analyses the average estimated thiamine concentration was 152.3  $\gamma$  per liter, with a range of 51.0 to 291.6  $\gamma$  per liter. The standard deviation of the differences between duplicates was  $\pm 7.02$   $\gamma$  per liter, or  $\pm 4.6$  per cent of the mean. This is probably an overestimation of the true replicate variance because simultaneity of the duplicate analyses was not scrupulously maintained in the series.

A more recent series of nineteen urine samples was examined with duplicate analyses on the day of collection and again in duplicate on the following day (storage at 5°). In this series the grand average thiamine concentration was 141.4  $\gamma$  per liter, with a range of 53 to 329 and a standard deviation about the grand mean of 84.4  $\gamma$  per liter. The standard deviation between duplicates was  $\pm 2.48$   $\gamma$  per liter, or 3.26 per cent of the mean. The variation of the differences between the duplicates was similar on the 2 separate days, but the total variation of replication plus storage for the 76 analyses was  $\pm 4.09$  per cent of the mean, and the difference between this value and that for simple replication was significant at the 2.5 per cent level. The additional variation introduced by the 24 hours of storage was separately calculated and proved to be  $\pm 2.47$  per cent of the mean. It should be noted that this was a random variation and that there was no systematic shift of level.

#### DISCUSSION

There is as yet no final criterion by which the absolute accuracy of a thiamine estimation in urine can be checked, and that limitation is recognized in the present work. It seems clear that the present method does eliminate certain errors which frequently are present in the ordinary procedure. The elimination, in large part at least, of non-thiamine fluorescent materials which are quenched or destroyed by ferricyanide makes it possible to obtain a more acceptable blank and to apply the calculation from the blank with greater security. At the same time the apparent thiochrome value is increased in many urines. We believe that these higher values are more nearly correct for several reasons: (1) the urine blanks are so low as to constitute only a minor correction in all cases; (2) thiamine added to urine is completely recovered in contradistinction to the results with many urines by the ordinary procedure; (3) with the present method mixtures of high and low blank urines give the

theoretical result, whereas low yields are obtained with the ordinary method; (4) negative thiamine values do not occur with this method, but they do occur with the ordinary method.

We have little information about the interfering fluorescent substances in urine. It does not appear that  $F_2$  is an important item in this category under normal circumstances. There may be other substances present which contribute to the total fluorescence read as thiochrome. If this is true it must be noted that their properties are so similar to thiamine as almost to defy separation, at least at present. Such a non-thiamine substance would have to possess the following properties: (1) be adsorbed on permutit; (2) be eluted with KCl-HCl; (3) be extracted from KCl-HCl with isobutanol; (4) show little or no fluorescence in the native state; (5) develop increased fluorescence in the presence of ferricyanide. Some foods, notably Sanka coffee, contain substances that conform to most of these characteristics, but they do not develop increased fluorescence with ferricyanide and are therefore readily distinguished.

Mason and Williams (3) attempted to use a blank prepared by treating urine with hydrosulfite. The addition of hydrosulfite has been criticized properly by Najjar and Ketron (6); it certainly destroys other substances besides thiamine, notably  $F_2$ . The procedure of Najjar and Ketron (6) has already been noted. Their reported results are deceptive in that true recoveries were not done. Najjar and Ketron first filtered urine through activated charcoal and then added known amounts of thiamine in order to test recovery. Undoubtedly the charcoal removed many potentially interfering substances. We have tested their method by true recovery experiments in which thiamine was added to the untreated urine and then their procedure followed exactly. Under these conditions the recoveries have been little if any better than with the ordinary procedure.

In many cases the difference in results between the present method and the ordinary method is small and even insignificant. Even in so called normal urines, however, there may be large differences which would result in a quite different diagnosis as to nutritional status by the urinary excretion criterion. For example, one person excreted 150  $\gamma$  of thiamine in 24 hours, according to the present method, but by the old method this person would have been considered deficient in thiamine, since only 86  $\gamma$  of thiamine were indicated (4). We have noted that in severe athiaminosis the ordinary method frequently gives meaningless results. The present method is certainly indicated in all cases in which low thiamine excretion is expected.

It is not suggested that all problems of thiamine estimation in urine have been resolved. The effect of volume is most puzzling. There are

some peculiar effects of variation in the intensity of the exciting radiation. Work on both these points is continuing in this laboratory.

#### SUMMARY

1. Human urine contains non-thiamine fluorescent substances which are not properly eliminated, either physically or by calculation, in the ordinary thiochrome methods. These interfering substances cause obvious gross errors in urines from athiaminotic persons and significant errors in many normal urines.

2. A thiochrome method is presented which eliminates all or most of these interfering substances by the control of acidity at the final extraction stage with isobutanol. The details are given for analyses on 5 ml. samples.

3. The present method never yields negative values, allows satisfactory recovery of added thiamine, and produces theoretical results in mixtures which have been separately analyzed. Previous thiochrome methods fail in these particulars with urines from persons on very low thiamine intakes.

4. Statistical evaluation of the present method has been made. In thirty-three pairs of duplicate samples the replicate standard deviation was 4.6 per cent of the mean.

5. The present method tends to yield higher thiamine values in urine than previous methods, especially in cases of thiamine deficiency. Diet and food items yield the same values with all methods tested.

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## LETTERS TO THE EDITORS

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### EFFECT OF BICARBONATE AND DIPHOSPHOPYRIDINE NUCLEOTIDE (DPN) ON DEHYDROGENASE ACTIVITY IN LIVER EXTRACTS

*Sirs:*

The dehydrogenase activity as measured by the rate of decolorization of methylene blue is considerably lower in dialyzed than in freshly prepared tissue extracts, a difference due largely to the loss of coenzyme factors and in particular to that of the pyridine nucleotides.<sup>1</sup> In the attempt to restore to dialyzed extracts of mouse liver the dehydrogenase activity approximating that of the freshly prepared extract, it was found that the addition of DPN to the dialyzed extract not only did not augment the dehydrogenase activity but actually depressed it. Only when bicarbonate was added together with DPN was there a striking increase in the activity. Bicarbonate added alone to the dialyzed extract caused a definite increase in activity but to no such magnitude as that reached in the presence of DPN. Both components are apparently needed for the restoration of dehydrogenase activity. The bicarbonate effect is relatively specific for this ion. Because of the relation noted between bicarbonate and pyruvate in recent reports<sup>2</sup> the effect of pyruvate addition was studied. This substrate first depressed the dehydrogenase activity, but on incubation with DPN and bicarbonate before the dye was added the activity considerably increased. Repeated experiments were entirely consistent and representative data are given in the table. Further experiments revealed that at higher concentrations of pyruvate with  $\text{NaHCO}_3$ -DPN and with longer periods of incubation the

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*Effect of Bicarbonate, DPN, and Pyruvate on Decolorization Rate of Methylene Blue in Dialyzed Extracts of Mouse Liver\**

Components of mixture†	Decolorization rate after standing‡	
	0 hr.	2 hrs.
	<i>min.</i> <sup>-1</sup> × 10 <sup>4</sup>	<i>min.</i> <sup>-1</sup> × 10 <sup>4</sup>
1 cc. NaCl-NaOH + 1 cc. H <sub>2</sub> O .....	10§	9
1 " NaHCO <sub>3</sub> + 1 cc. H <sub>2</sub> O .....	24	24
1 " NaCl-NaOH + 1 cc. DPN .....	5	5
1 " NaHCO <sub>3</sub> + 1 cc. DPN .....	840	840
1 " NaCl-NaOH + 1 cc. pyruvate .....	11	11
1 " NaHCO <sub>3</sub> + 1 cc. pyruvate .....	24	33
1 " NaHCO <sub>3</sub> -DPN + 1 cc. pyruvate¶ .....	385	1000-3000

\* Aqueous extract of mouse liver (equivalent to 111 mg. of tissue per cc.) dialyzed 48 hours at 0° against distilled water.

† Stock solutions, NaHCO<sub>3</sub> 0.04 M; DPN 1.12 mg. per cc. of H<sub>2</sub>O; sodium pyruvate  $1.6 \times 10^{-3}$  M; NaCl-NaOH 0.04 N in respect to sodium ion and containing enough NaOH to give the same pH, namely 8.0, in the mixture containing tissue extract as do the same mixtures with NaHCO<sub>3</sub>; NaHCO<sub>3</sub>-DPN 1.12 mg. of the nucleotide per cc. of 0.04 M NaHCO<sub>3</sub>; methylene blue  $0.31 \times 10^{-3}$  M. Solutions in the dehydrogenation vessels were composed of 1 cc. of tissue extract + 1 cc. of methylene blue solution + the volumes of components designated, a total of 4 cc. in each case. Temperature 26°. Technique of measurement according to Chalkley and Greenstein (*J. Nat. Cancer Inst.*, **6**, in press).

‡ Mixtures were prepared in replicate of the tissue extract with the components designated, with the methylene blue in the side arm. The dehydrogenation tubes were evacuated at 18 mm. of Hg for 2 minutes and sealed. 0 hour refers to tubes into which dye is tipped immediately after sealing. 2 hours refers to tubes into which dye is tipped 2 hours after sealing. Temperature during this interval, 26°.

§ The rate under these conditions for freshly prepared mouse liver extract is approximately 300.

|| The rate under these conditions for freshly prepared mouse liver extract is approximately 1100.

¶ Addition of 0.001 M Mn<sup>++</sup> was somewhat retarding.

effect was even more pronounced. The stimulating effect of bicarbonate has been observed in other cases.<sup>3</sup>

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# REACTION OF THE ISOCITRIC DEHYDROGENASE SYSTEM WITH CYTOCHROME *c*\*

Sirs:

Since, as shown by Adler *et al.*,<sup>1</sup> isocitric dehydrogenase reacts specifically with triphosphopyridine nucleotide (TPN), and since cytochrome reductase<sup>2</sup> catalyzes the reoxidation of reduced TPN by cytochrome *c*, cytochrome reductase should link the isocitric dehydrogenase system with

## *Reduction of Ferricytochrome c by Isocitric Dehydrogenase System in Presence of Cytochrome Reductase*

Concentration of reactants (moles  $\times 10^{-8}$  per cc.): *l*-isocitric acid 17.5,  $\text{MnCl}_2$  60.0, TPN 0.5, cytochrome *c* 3.79. Isocitric dehydrogenase, 25  $\gamma$  of protein per cc. Gas, air; temperature, 28°;  $\lambda$ , 550  $\mu$ ;  $d = 0.5$  cm.

Cytochrome reductase	Ferricytochrome c, moles $\times 10^{-8}$ per cc				$\Delta \log$ ferricytochrome c in 1st min
	Initial	After addition of cytochrome reductase			
		1 min	2 min.	4 min	
$\gamma$ per cc.					
17.5	3.79	3.21	2.78	2.37	0.07
35.0	3.79	2.75	1.90	1.11	0.14*
52.5	3.79	2.23	0.94	0.22	0.23
70.0	3.79	1.91	0.51		0.30

\* The same reaction rates were obtained with  $3.5 \times 10^{-8}$  mole per cc. of isocitric acid, or with an amount of isocitric dehydrogenase giving a final concentration of 5  $\gamma$  of protein per cc. There was no increase in rate when the TPN concentration was doubled.

cytochrome *c*. The accompanying table shows that such is the case. The concentration of ferricytochrome *c* was determined spectrophotometrically at 550  $\mu$  and calculated by the equation given by Haas *et al.*<sup>2</sup>

The isocitric dehydrogenase used was a purified preparation from pig heart obtained by salt fractionation; it was free from aconitase but still contained oxalosuccinic carboxylase.<sup>3</sup> In the presence of  $\text{Mn}^{++}$  isocitric acid is converted to  $\alpha$ -ketoglutaric acid and  $\text{CO}_2$  by this preparation. The

\* Supported by grants from the Rockefeller Foundation, the Penrose Fund of the American Philosophical Society, the Williams-Waterman Fund of the Research Corporation, and Hoffmann-La Roche, Inc.

<sup>1</sup> Adler, E., von Euler, H., Günther, G., and Plass, M., *Biochem. J.*, **33**, 1028 (1939).

<sup>2</sup> Haas, E., Horecker, B. L., and Hogness, T. R., *J. Biol. Chem.*, **136**, 747 (1940).

<sup>3</sup> Ochoa, S., and Weisz-Tabori, E., *J. Biol. Chem.*, **159**, 245 (1945).

cytochrome reductase was a partially purified preparation kindly supplied by Mr. Kurt Altman.<sup>4</sup>

In the experiments reproduced in the table all reactants were present in excess relative to cytochrome reductase, so that the concentration of the latter was the rate-limiting factor. With the lower concentrations of reductase the reduction of cytochrome *c* was of the first order; the initial rate was proportional to the reductase concentration in all cases. Without isocitric acid, isocitric dehydrogenase, TPN, or cytochrome reductase there was no reaction.

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<sup>4</sup> Spectrophotometric estimation at 455  $m\mu$  indicated a purity of 17 per cent, but, judging by the activity of pure cytochrome reductase in the experiments of Haas *et al.*, probably less than 20 per cent of the flavoprotein thus determined was cytochrome reductase.

## FIXATION OF CARBON DIOXIDE IN OXALACETATE BY PIGEON LIVER

Sirs:

The fixation of  $\text{CO}_2$  in oxalacetate as shown in Reaction 1 has previously been demonstrated with a bacterial preparation.<sup>1</sup>



A similar attempt with pigeon liver failed,<sup>2</sup> although fixation of  $\text{CO}_2$  in  $\alpha$ -ketoglutarate<sup>3,4</sup> and in fumarate and lactate<sup>2,4</sup> with this tissue had been well established. We have found that in the presence of adenosine triphosphate (ATP), Reaction 1 occurs readily with liver extracts, thus demonstrating the primary fixation reaction in animal tissue.

In the table it is shown that some  $\text{C}^{13}\text{O}_2$  is fixed in the carboxyl adjacent to the methylene group in oxalacetate without ATP but that addition of the latter greatly increases the  $\text{C}^{13}$  fixed. In this experiment, an undialyzed extract obtained from an acetone powder of pigeon liver similar to that described by Evans *et al.*<sup>5</sup> was used. The extract was incubated with oxalacetate, pyruvate,  $\text{MnCl}_2$ , and  $\text{NaHC}^{13}\text{O}_3$  until about one-half of the oxalacetate had been decarboxylated. Occasionally, undialyzed preparations may be quite active in fixation even in the absence of ATP, but ATP is always necessary for effective fixation with dialyzed preparations.

According to Reaction 1,  $\text{C}^{13}\text{O}_2$  should be found only in the carboxyl adjacent to the methylene group in oxalacetate unless the latter compound is in equilibrium with a symmetrical substance such as fumarate. Since the  $\text{C}^{13}$  was fixed only in the one position, the possibility is eliminated that the initial fixation reaction involved fumarate or any substance in equilibrium with fumarate. The carboxyl adjacent to the methylene groups was obtained by acid heat decarboxylation and the second carboxyl, along with the pyruvate carboxyl, by treatment with ceric sulfate. The effectiveness of the removal of residual  $\text{C}^{13}\text{O}_2$  preliminary to the degradation of oxalacetate is attested by the low  $\text{C}^{13}$  values of the  $\text{CO}_2$  collected from the last portion of the  $\text{CO}_2$  rinse used in removal of  $\text{C}^{13}\text{O}_2$  from the medium.

<sup>1</sup> Krampitz, L. O., Wood, H. G., and Werkman, C. H., *J. Biol. Chem.*, **147**, 243 (1943).

<sup>2</sup> Wood, H. G., Vennesland, B., and Evans, E. A., Jr., *J. Biol. Chem.*, **159**, 153 (1945).

<sup>3</sup> Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).

<sup>4</sup> Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 31 (1942).

<sup>5</sup> Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771 (1943).

*Fixation of  $C^{13}O_2$  in Oxalacetate*Values as per cent excess  $C^{13}$ .

Experiment No.	Adenosine triphosphate	Oxalacetate		Rinse
		Carboxyl adjacent to methylene group	Carboxyl adjacent to carbonyl group	
1	<i>M</i>	0.069	0.005	0.019
2	0.007	0.246	0.005	0.008

Each experiment was conducted anaerobically for 45 minutes at 38° and contained 3 ml. of undialyzed liver extract, 1.0 mm of oxalacetate, 0.4 mm pyruvate, 0.05 mm  $MnCl_2$ , 0.4 mm phosphate buffer, and 0.7 mm  $NaHC^{13}O_3$  (excess  $C^{13}$  = 4.64 per cent) in a total volume of 15 ml.

It is not clear at the present time whether ATP participates directly in the fixation reaction or is involved in a secondary rôle. Other experiments indicate that adenylic acid will not replace ATP.

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# ON THE DETERMINATION OF CHOLINE IN THE LIVER AND PLASMA OF THE DOG\*

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It was found in this laboratory that practically all phospholipids of plasma of the dog and man are of the choline-containing type, an observation that is not in agreement with those of other investigators (1). It was pointed out that the explanation of the discrepancy between the results obtained here and elsewhere would probably be found in the methods employed for the determination of phospholipid choline. In the method used in this laboratory the reineckate of choline is precipitated in an acid medium (2). Glick, in a recent communication, states, however, that in order to avoid the precipitation by reineckate of compounds other than choline it is necessary to perform this precipitation in an alkaline medium (3). This worker compared the amount of reineckate precipitated at pH 8 to 10 and at pH 2 to 3 in extracts of hydrolyzed wheat germ, soy bean, yeast, calf liver, and hog brain. In the case of wheat germ and yeast, he found significant differences in the amounts of reineckate precipitated at these two pH ranges. In the case of calf liver and hog brain, the values obtained for each tissue at both pH ranges were strikingly similar when 10 per cent KOH was used for hydrolysis. When saturated barium hydroxide was employed for hydrolysis, the values obtained for hog brain at the two pH ranges were in good agreement; a difference, however, was observed for calf liver.

In view of the above considerations, it became of interest to compare the choline contents of plasma and liver phospholipids as determined by the method of Entenman *et al.* (2) and of Glick (3). This has been done in the present investigation.

## EXPERIMENTAL

The following extracts were prepared from plasma and liver of the dog.

### *Plasma Extracts*

*Alcohol-Ether Extracts*—Plasma was extracted with 30 volumes of 3:1 alcohol-ether mixture at 55° for 1 hour. The mixture was then filtered and the residue extracted with ether for 8 hours in a Soxhlet apparatus.

\* Aided by a grant from the Lederle Laboratories, Inc.

The ether and alcohol-ether extracts were combined, filtered, and made to volume.

*Solution of Precipitated Phospholipids*—A measured volume of the alcohol-ether extract described above was concentrated at 55° to a small volume (about 1 cc.) under reduced pressure in an atmosphere of CO<sub>2</sub>. The lipids of this concentrate were extracted with several portions of petroleum ether. The latter was then reduced to a small volume of approximately 1 cc. by boiling and the phospholipids precipitated by the addition of 30 cc. of acetone and 10 to 15 drops of a saturated solution of MgCl<sub>2</sub> in absolute alcohol. The mixture was centrifuged and the supernatant discarded. The precipitate was washed with acetone. It was then suspended in 2 cc. of ethyl ether and the phospholipids brought into solution by the addition of methanol. This ether-methanol solution was then made to volume.

*Methanol Extract*—Separate samples of plasma were extracted with 30 volumes of methanol for 1 hour at 55°. The extract was filtered and the residue washed with methanol. The extract and washings were made to volume.

*Acetone Extract*—5 cc. of plasma were slowly transferred to a 50 cc. centrifuge tube containing 20 cc. of acetone; the latter was vigorously agitated with a glass rod during the transfer. The mixture was allowed to stand at room temperature for 30 minutes and then centrifuged. The supernatant was transferred to a volumetric flask and made to volume.

### *Liver Extracts*

*Alcohol-Ether Extract*—Weighed samples of liver were ground in a mortar and extracted with ethyl alcohol at 55° for 2 hours. Approximately 150 cc. of alcohol were used for 20 to 25 gm. of liver. The supernatant was decanted through a filter paper and the residue extracted with a second portion of alcohol for 1 hour. The contents of the flask were then poured through the same filter paper and the two alcohol extracts combined. The tissue residue was then extracted overnight with ether in a Soxhlet apparatus and the ether extract added to the alcohol extracts. The alcohol and ether extracts were combined and made to a convenient volume.

*Solution of Precipitated Phospholipid*—Aliquots of an alcohol-ether extract prepared as described above were concentrated to a volume of about 1 cc. under reduced pressure in an atmosphere of CO<sub>2</sub>. The lipids were removed from this concentrate with petroleum ether and the phospholipids precipitated from its petroleum ether solution as described above for plasma. The precipitated phospholipids were then redissolved in ethyl ether and methanol and made to volume.

### *Methods of Analysis*

Choline was determined by two methods, that of Entenman *et al.* (2) and that of Glick (3). Both methods were slightly modified.

*Method of Entenman et al.*—The modification of the method of Entenman *et al.* consisted of the preparation of the barium hydroxide solution. 10 gm. of  $\text{Ba}(\text{OH})_2$  were dissolved in 100 cc. of hot distilled water and the solution formed was filtered. 15 cc. of this solution were used for each hydrolysis instead of 15 cc. of a saturated solution of barium hydroxide as originally described.

*Glick's Method*—The hydrolysis was carried out as follows. An aliquot of an extract was added to a 125 cc. Erlenmeyer flask and the volume reduced to about 5 cc. by evaporation on a steam bath. 15 cc. of the barium hydroxide solution prepared as described above were added to each flask and a bubble stopper placed on the flask. The mixture was then heated for 2 hours by direct contact with steam. After cooling, 1 drop of thymolphthalein indicator was added and this was followed by the addition of glacial acetic acid, until the blue color just disappeared. The mixture was then filtered with suction through a medium sized fritted glass filter and the residue washed twice with distilled water. The filtrate was collected in a 30 cc. glass vial placed inside the suction flask.

Choline was precipitated by the addition to the filtrate of a solution of the Reinecke salt in methanol, as described by Glick. The mixture was allowed to stand for 2 hours and then filtered through a fritted glass filter of medium porosity and the residue washed with 2.5 cc. of *n* propanol. The precipitate was dissolved in acetone, the acetone solution passed through the glass filter, and the filtrate collected in a 15 cc. centrifuge tube that had been placed inside the suction flask. The contents of the centrifuge tube were mixed thoroughly with a glass rod, the volume read, and the solution transferred to a colorimeter tube and stoppered. The color was determined by the Klett-Summerson photoelectric colorimeter, as previously described (2).

Phosphorus was determined on aliquots of the various extracts by King's method (4).

### *Results*

*Plasma*—The following fractions of dog plasma prepared as described above were analyzed for choline and phosphorus. (1) An alcohol-ether extract, (2) an ethyl ether-methanol solution of phospholipids that had been precipitated from a petroleum ether extract of plasma, (3) a methanol extract, and (4) an acetone extract. The results are recorded in Table I.

The choline contents of the alcohol-ether extracts of the plasma of seven



TABLE I  
*Comparison of Values Obtained for Choline Contents of Various Extracts of Dog Plasma by Methods of Glick and of Entenman et al.*

Dog No.	Choline method	Alcohol-ether extract				Pptd. phospholipids				Methyl alcohol extract				Acetone extract			
		Choline	Phos-phorus	Choline	Choline	Phos-phorus	Choline	Phos-phorus	Choline	Choline	Phos-phorus	Choline	Choline	Choline	Phos-phorus	Choline	Choline
		mg. per 100 cc.	mg. per 100 cc.	molal ratio	mg. per 100 cc.	mg. per 100 cc.	molal ratio	mg. per 100 cc.	mg. per 100 cc.	molal ratio	mg. per 100 cc.	mg. per 100 cc.	molal ratio	mg. per 100 cc.	mg. per 100 cc.	molal ratio	mg. per 100 cc.
9	Entenman et al.	44.8	11.1	1.03	41.5	11.1	0.96	47.5	11.3	1.07	40.7	9.8	1.07	40.7	9.8	1.07	40.7
	Glick	43.7		1.01	42.5		0.99	44.5		1.01	38.1		1.01	38.1		1.00	38.1
10	Entenman et al.	48.0		1.17	41.5		1.00	44.5		1.07	41.5		1.07	41.5		1.04	41.5
	Glick	48.0	10.5	1.17	43.0	10.6	1.04	44.0	10.7	1.06	41.0	10.2	1.06	41.0	10.2	1.03	41.0
11	Entenman et al.	60.5		1.06	55.8		1.04	60.5		1.00	55.5		1.00	55.5		1.07	55.5
	Glick	58.0	14.6	1.02	54.2	13.7	1.01	61.5	14.3	1.02	54.0	13.1	1.02	54.0	13.1	1.05	54.0
1*	"	28.0	7.40	0.97	29.0	7.45	1.00	30.8	7.75	1.02			1.02				
2*	"	35.3	9.85	0.92	34.2	9.40	0.94	35.0	10.3	0.82			0.82				
3*	"	89.0	23.2	0.98	88.5	23.5	0.97	90.5	24.7	0.94			0.94				
12	Entenman et al.	48.5	12.4	1.00	45.7	12.4	0.95										
	Glick	48.2		0.99	46.0		0.95										
13	Entenman et al.	34.0	8.75	1.00	30.0	7.50	1.00	30.2	7.50	1.00			1.00				
	Glick	34.4		1.00	30.2		1.00			1.00			1.00				
14	Entenman et al.	37.5	9.8	0.98	37.5	9.3	1.03	37.0	9.3	1.03			1.03				
	Glick	40.0		1.04	37.0		1.02			1.02			1.02				
15	Entenman et al.	64.5	15.9	1.04	61.0	16.1	0.98	61.0	16.1	0.98			0.98				
	Glick	61.5		0.99	61.0		0.98			0.98			0.98				

\* Dogs 1, 2, and 3 had been depancreatized 5 months before plasma samples were taken for analyses; they were maintained on a lean meat-sucrose diet and injected twice daily with 8 units of insulin. All the other dogs were normal.

dogs were measured by the methods of Entenman, Taurog, and Chaikoff (2) and of Glick (3). The two values obtained for each of three plasma samples of Dogs 10, 12, and 13 were practically identical. The two values obtained for each of the other four plasma samples were also in good agreement. Thus the maximum difference was observed in the two values for Dog 15; these were 64.5 and 61.5 mg., the latter having been obtained by the Glick method.

The choline contents of plasma phospholipids that had been precipitated by means of acetone and  $MgCl_2$  were determined by the two methods and the values recorded in Table I. The agreement between the two values obtained for each of the seven plasma samples was exceedingly good.

The choline contents of methanol extracts of three samples of plasma were compared by the two methods. The values obtained for two of the three extracts were practically identical.

In Table I, two choline values are shown for each of three acetone extracts of plasma. In a single sample (Dog 9) a difference of 2.6 mg. was observed between the two values obtained by the methods of Glick and of Entenman *et al.* But the two values obtained for the other two dogs (Nos. 10 and 11) agreed well.

Table I shows the amounts of choline and phosphorus that can be extracted from plasma by various types of solvents or procedures. The methanol and the alcohol-ether extracts contained the highest amounts of choline as judged by either method of estimation of choline. It is of interest to note here that extraction with alcohol and ether removes the same amount of choline from plasma as does treatment with methanol alone. These two extracts, however, contained only slightly more choline than did the fraction designated here as "precipitated phospholipids." As was to be expected, the lowest amounts of choline were found in the acetone extracts.

The highest values for phosphorus were consistently found in the methanol extracts and the lowest in the acetone extracts. Good agreement, however, was observed between the phosphorus values of an alcohol-ether extract and of the fraction designated "precipitated phospholipids."

*Liver*—Two fractions of dog liver were analyzed for choline and phosphorus, (1) an alcohol-ether extract and (2) an ethyl ether-methanol solution of phospholipids that had been precipitated as described above.

Alcohol-ether extracts were prepared from the livers of seven dogs and their choline contents, as measured by the methods of Glick and of Entenman *et al.*, compared (Table II). In only two of the extracts was good agreement observed between the two choline values (Dogs 4 and 7). In the other five extracts higher values were obtained by Entenman's method than by Glick's. Thus in the case of the liver extract of Dog 1, a difference

of 20 per cent was observed between the choline values as measured by the two methods. A similar difference was found in the alcohol-ether extract of the liver of Dog 3.

The two values found for the choline content of phospholipids that had been isolated from a petroleum ether extract of liver by precipitation with acetone and  $MgCl_2$  were in good agreement. In six of the eight samples analyzed the difference between the two values did not exceed 4 per cent; the maximum difference was 10 per cent.

TABLE II

*Comparison of Values Obtained for Choline Content of Extracts of Dog Liver by Methods of Glick and of Entenman et Al.*

Dog. No.*	Choline method used	Alcohol-ether-soluble			Phospholipid		
		Choline	Phos- phorus	Choline P	Choline	Phos- phorus	Choline P
		mg. per 100 cc.	mg. per 100 cc.	molar ratio	mg. per 100 cc.	mg. per 100 cc.	molar ratio
1	Entenman <i>et al.</i>	175	82	0.55	148	78	0.49
	Glick	139		0.43	134		0.44
2	Entenman <i>et al.</i>	139	71	0.50	111	67	0.42
	Glick	112		0.40	109		0.42
3	Entenman <i>et al.</i>	224	83	0.69	186	76	0.63
	Glick	182		0.56	188		0.63
4	Entenman <i>et al.</i>	308	139	0.57	274	132	0.54
	Glick	294		0.54	268		0.52
5	Entenman <i>et al.</i>	340	145	0.60	264	122	0.56
	Glick	325		0.58	258		0.54
6	Entenman <i>et al.</i>	344	146	0.60	320	139	0.59
	Glick	330		0.60	298		0.55
7	Entenman <i>et al.</i>	335	147	0.58	306	139	0.56
	Glick	334		0.58	296		0.55
8	Entenman <i>et al.</i>				158	62	0.65
	Glick				151		0.62

\* See the foot-note to Table I.

The results presented in Table II show that the choline content of an alcohol-ether extract of the liver *may* be much higher than that of the fraction designated precipitated phospholipids. This was consistently the case *only* when the method of Entenman *et al.* was used for the determination of choline. Thus, as judged by both methods, the amount of choline found to be alcohol-soluble in the livers of Dogs 4, 5, 6, and 7 was much greater than that found as phospholipids. But in Dogs 1, 2, and 3 the choline values of the two fractions as measured by the Glick method agreed quite closely, whereas when choline was measured by the method of Enten-

man *et al.* the alcohol-ether-soluble choline was as much as 30 per cent higher than phospholipid choline.

The phosphorus content of the solution of precipitated phospholipid was usually about 5 per cent less than that of the alcohol-ether extract.

### *Comment*

The results of the present investigation show that identical choline values are obtained for plasma and liver phospholipids whether the choline reineckate is precipitated in the presence of 1.2 N HCl (method of Entenman *et al.*) or at a pH between 8 and 9 (Glick's method). The phospholipid analyzed in these experiments represents a fraction that had been precipitated from petroleum ether extracts of liver and plasma by acetone and  $MgCl_2$ . The same choline values were also obtained by these two methods when analysis was made of an extract of plasma designated here "alcohol-ether soluble."

A definite difference did appear, however, in the choline values of an alcohol-ether extract of liver; in this case lower values were found when Glick's method was used. This may mean, as Glick points out, that in an alkaline medium the precipitation of compounds other than choline by reineckate is less likely to occur. The fact that, in contrast to the alcohol-ether extract of liver, identical values were found for phospholipid choline is not surprising, since the phospholipids used for analysis here were first precipitated from a petroleum ether solution by the addition of acetone and  $MgCl_2$  and subsequently washed with acetone, a procedure that served to free the phospholipids from the possible presence of compounds that form insoluble reineckates.

It is of interest to note here that the ratios of choline to phosphorus for plasma phospholipids were found to be unity or quite close to unity when choline was determined by Glick's method as well as when the method of Entenman *et al.* was used. The earlier observation that all phospholipids of dog plasma are of the choline-containing type is therefore confirmed (1).

The values observed for the choline content of the methyl alcohol-soluble fraction of plasma were somewhat higher than those for the other three fractions studied. Interestingly enough, the choline content of the precipitated phospholipid fraction is but little less than that of the methyl alcohol extracts. The latter contained from 2 to 13 per cent (average 6) more choline than the phospholipid fraction. If allowance is made for the loss of phospholipids, probably less than 5 per cent, that occurs during their isolation, precipitation, and subsequent solution in a mixture of methanol and ether, it would appear that nearly all the choline contained in the methyl alcohol extract had been derived from plasma phospholipids. If, in addition, it is assumed that the choline content of the methyl alcohol

extract represents the total choline content of plasma, then the results presented here lead to the conclusion that dog plasma contains very little choline that is not bound as phospholipid. It can be estimated that less than 5 per cent of plasma choline exists in the free state.

Further evidence that practically no free choline exists in plasma is provided by the data shown in Table III. Choline chloride was added to dog plasma and its recovery determined in an alcohol-ether extract of plasma and in a fraction of plasma designated as precipitated phospholipids. The preparation of each has been described in the section on plasma extracts. Practically all the added choline was recovered in the alcohol-

TABLE III

*Recovery of Choline Added to 5 Cc. of Dog Plasma in Its Alcohol-Ether Extract and in Its Precipitated Phospholipid Fraction*

Plasma sample No.	Choline added to plasma	Alcohol-ether extract of plasma			Pptd. phospholipid fraction of plasma		
		Total choline found	Recovery of added choline		Total choline found	Recovery of added choline	
	mg.	mg.	mg.	per cent	mg.	mg.	per cent
1	0	2.26			2.16		
2	0	2.25			2.16		
3	0	2.23					
4	0	2.29					
5	1.92	4.09	1.83	96.5			
6	1.92	4.15	1.89	98.5			
7	1.92	4.12	1.86	97.0			
8	1.92	4.06	1.80	94.0			
9	1.92				2.18	0	0
10	1.92				2.12	0	0

ether extract. None of it was found in the phospholipid fraction. There can be no doubt, therefore, that the phospholipid fraction as prepared here does not retain free choline.

The above conclusions on the amounts of *free* choline contained in *dog* plasma are not in line with the views of Luecke and Pearson (5), who report that from 24 to 31 per cent of the choline contained in the plasma of the horse, beef, and sheep, and as much as 26 per cent of human plasma, exists in the free form. These workers claim to have effected a separation of plasma choline into free and combined merely by the addition of acetone; they regard acetone-soluble choline as free choline. In reviewing the question of solvents for phospholipids, Thierfelder and Klenk (6) pointed out in 1930 that the amount of phospholipid from fresh organs soluble in acetone may be considerable. Luecke and Pearson (7) have shown that a water solution of soy bean lecithin can be completely precipitated by acetone, but it is not surprising that the solubility properties of phospho-

lipids as they exist in fresh tissue differ from those of isolated lecithin that has been subjected to various chemical procedures necessary for its isolation.

In order to test the solubility of plasma phospholipids under the conditions described by Luecke and Pearson (5) 5 cc. of plasma were added to 20 cc. of acetone and the resulting precipitate removed by centrifugation. Choline and phosphorus were measured in the supernatant fluid and the results recorded in Table I. Most of the choline and phosphorus of plasma remains in the acetone when 1 volume of plasma is mixed with 4 volumes of acetone. Since the ratio of choline to phosphorus of the acetone supernatant is approximately unity, it is reasonable to conclude that the choline found to be soluble in acetone was phospholipid choline.

#### SUMMARY

Various extracts and fractions of plasma and liver were analyzed for choline by the methods of Glick (3) and of Entenman *et al.* (2).

1. Identical choline values were found for the phospholipid fraction isolated from either liver or plasma when the precipitations of choline reprecipitate were carried out (1) in the presence of 1.2 N HCl (method of Entenman *et al.*) and (2) at a pH between 8 and 9 (Glick's method).

2. The values obtained when the two methods were used for the estimation of the choline content of an alcohol-ether extract of plasma were in good agreement. Both methods also gave identical values when a methanol extract of plasma was analyzed.

3. When an alcohol-ether extract of liver was examined, the choline value as measured by the Glick method was found to be lower than that obtained by the method of Entenman *et al.*

4. The alcohol-ether extract and the phospholipid fraction of plasma contained the same amounts of choline and phosphorus.

5. Practically all the choline contained in the methanol and alcohol-ether extracts of plasma is bound as phospholipids. It is estimated that less than 5 per cent of plasma choline is in the free form.

6. The earlier finding that practically all phospholipids of dog plasma are choline-containing is confirmed.

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# THE MECHANISM OF ACTION OF THE ANTIFATTY LIVER FACTOR OF THE PANCREAS\*

## I. ITS RELATION TO PLASMA CHOLINE

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The accumulation of fat in the liver of the completely depancreatized dog maintained with insulin results from the loss of the external secretion of the pancreas. This was shown by two findings: fatty livers can be induced by ligation of the pancreatic ducts (1-3); and the development of fatty livers can be prevented by the feeding of pancreatic juice, not only in the duct-ligated dog but also in the completely depancreatized dog kept alive with insulin (4).

Although the mechanism by which the exclusion of the external secretion of the pancreas produces fatty livers is not known, the following observations are pertinent to its understanding. Fatty livers develop in dogs deprived of the external secretion of the pancreas, despite the fact that such dogs are fed a diet that is not deficient in choline or in methyl, but can be prevented by the addition of *extra* free choline to the diet or by the ingestion of fractions of the pancreas (5, 6). It has been clearly established that it is not the choline content of the pancreas fraction fed that accounts for its antifatty liver action. The ingestion of as little as 60 mg. per day of Fraction 27C, which is free of choline, is sufficient to prevent fatty livers in completely depancreatized dogs weighing 10 to 12 kilos (6), whereas the minimum effective dose of choline was found to be 35 mg. per kilo per day (5).

In 1934 it was reported that the increased fat content of the livers of depancreatized dogs receiving insulin is accompanied by decreased amounts of phospholipids in their blood (7). A similar decrease in the phospholipid level of the plasma was later observed in dogs in which the external secretion of the pancreas was excluded from the intestinal tract (8). A recent analysis of plasma phospholipids shows them to be of the choline-containing type (9); it was estimated that less than 5 per cent of the phospholipids of plasma is of the non-choline-containing type. Moreover, according to the findings of the preceding paper, practically all choline of plasma is contained in the phospholipid molecule (10). The indications are therefore

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strong that the dog in which the flow of pancreatic juice into the intestinal tract has been interrupted is suffering from a deficiency of circulating choline.

The present report deals with the levels of plasma choline of depancreatized and duct-ligated dogs fed various diets and the response of these levels to the addition of fractions of the pancreas.

TABLE I

*Plasma Choline of Completely Depancreatized and Pancreatic Duct-Ligated Dogs Maintained on Meat-Sucrose Diet*

Dog No.	Weight	Time of observation	Plasma choline
	kg.		mg. per 100 cc.
D553	8.2	Before pancreatectomy	61.3
	7.0	8 wks. after pancreatectomy	46.6
	6.5	12 " " "	41.3
D552	6.0	Before pancreatectomy	62.0
	5.9	28 wks. after pancreatectomy	45.0
	5.4	52 " " "	40.0
D537	12.5	Before pancreatectomy	58.0
	12.4	16 wks. after pancreatectomy	37.2
D528	15.0	Before pancreatectomy	49.6
	7.8	20 wks. after pancreatectomy	25.1
D524	10.1	Before pancreatectomy	61.1
	7.0	29 wks. after pancreatectomy	20.6
	7.5	56 " " "	40.3
E91	11.0	Before ligation of pancreatic ducts	55.7
	8.1	22 wks. after ligation of pancreatic ducts	29.3
E92	9.6	Before ligation of pancreatic ducts	54.5
	8.4	20 wks. after ligation of pancreatic ducts	27.5
E93	12.7	Before ligation of pancreatic ducts	56.8
	8.5	12 wks. after ligation of pancreatic ducts	35.6
E94	11.7	Before ligation of pancreatic ducts	53.0
	7.2	20 wks. after ligation of pancreatic ducts	26.8
E95	15.5	Before ligation of pancreatic ducts	52.5
	7.4	14 wks. after ligation of pancreatic ducts	30.4

1. *Plasma choline is decreased in the completely depancreatized dog maintained with insulin and in the dog subjected to ligation of pancreatic ducts.* The preparation of dogs for pancreatectomy or ligation of their pancreatic ducts has been described elsewhere (11). Following either of these surgical procedures, each dog listed in Table I was fed twice daily 250 gm. of lean meat, 50 gm. of sucrose, 10 gm. of bone ash, and 1 gm. of Cowgill's salt mixture (12). Each dog also received once daily 3 cc. of sardilene and 5 gm. of yeast (11). Insulin was injected twice daily, immediately after the ingestion of the diet.

Plasma choline was measured on the day of operation and at various intervals after pancreatectomy or duct ligation. The method of Entenman *et al.* was used (13). The results are recorded in Table I. A significant reduction in plasma choline occurred as early as 8 weeks after pancreatectomy (Dog D553); the level of plasma choline was still below normal as late as 1 year after complete excision of the gland (Dogs D552 and D524).

The earliest observation on the level of plasma choline after duct ligation was made at an interval of 12 weeks (Dog E93), at which time the con-

TABLE II

*Effect of Prolonged Feeding of 1 Gm. of Pancreas Fraction AR\* on Plasma Choline of Depancreatized Dogs Fed Meat-Sucrose Diet*

Dog No.	Weight	Time of observation			Plasma choline
	kg.				mg. per 100 cc.
D533	6.6	3 wks.	before	pancreatectomy	67.0
	6.7	2	"	"	63.5
	6.7	0†	"	"	65.5
	6.2	15	"	after	66.5
	5.9	19	"	"	63.5
	6.0	25	"	"	73.0
D534	6.2	2	"	before	73.0
	5.8	0†	"	"	66.5
	5.9	6	"	after	77.0
	5.8	8	"	"	89.0
	6.0	11	"	"	77.0
	5.9	15	"	"	77.0
D535	6.5	22	"	"	92.0
	7.9	3	"	before	64.2
	8.0	0†	"	"	58.0
	7.5	11	"	after	72.6
	7.2	14	"	"	70.3
	7.4	22	"	"	70.8

\* The method of preparation of Fraction AR has been described (5).

† Day of pancreatectomy.

centration of plasma choline had fallen from a preoperative value of 57 to 36 mg. per 100 cc. The longest interval studied was 22 weeks (Dog E91), by which time the level of plasma choline was still considerably below the preoperative level.

2. *The daily feeding of 1 gm. of pancreas Fraction AR prevents the fall of plasma choline levels in completely depancreatized dogs fed a lean meat diet.* Dogs D533, D534, and D535 were depancreatized and then fed the diet described in Section 1. In addition each dog received once daily 1 gm. of Fraction AR, a pancreas fraction derived from 5.5 gm. of fresh tissue (5). It was shown earlier that the daily ingestion of 1 gm. of Fraction AR was

sufficient to prevent the development of fatty livers in completely depancreatized dogs (5).

TABLE III  
*Effect of Addition and Removal of Pancreas Fractions on Plasma Choline of Depancreatized Dogs Fed Meat-Sucrose Diet*

Dog No.	Weight	Pancreas fraction administered				Plasma choline
		Type	Interval fed	Total days fed	Amount fed daily	
	kg.				gm.	mg. per 100 cc.
D548	7.4	AR	Begun	20	2.0	41.2
	7.7		Stopped			63.0
	7.4					43.0
D560	6.5	AR	Begun	21	2.0	45.7
	7.4		Stopped			77.5
	6.3					49.2
D562	8.4	AR	Begun	21	2.0	23.8
	9.0		Stopped			53.5
	8.0					34.7
D502	7.5	AR	Begun	20	1.8	33.7
	7.8		Stopped			62.2
	7.2					37.0
D510	8.6	AR	Begun	21	1.0	35.0
	8.8		Stopped			59.0
	8.2					29.3
D561	7.6	AR	Begun	24	1.0	44.5
	8.3		Stopped			61.9
	7.5					35.5
					mg	
D511	8.2	27C*	Begun	21	60	47.5
	8.8		Stopped			65.3
	8.5					39.4
D513	4.4	27C	Begun	21	60	46.2
	4.6		Stopped			69.6
	4.5					47.0
D514	8.7	27C	Begun	21	60	59.4
	9.2		Stopped			73.5
	9.3					55.2
D548	7.5	27C	Begun	21	60	39.5
	7.7		Stopped			63.2
	7.0					36.8

\* The preparation of Fraction 27C has been described (6).

Plasma choline was determined repeatedly in these three dogs both before and after excision of the pancreas. The results recorded in Table II leave no doubt that the fall in plasma choline that occurs in depancreatized

dogs maintained on the lean meat-sucrose diet can be prevented by the addition of 1 gm. of Fraction AR per day. The levels of plasma choline found 22 to 25 weeks after pancreatectomy were as high as or higher than the preoperative levels.

3. *Pancreas fractions increase circulating choline when ingested by depancreatized dogs maintained with insulin and a lean meat diet.* Following pancreatectomy the dogs listed in Table III were fed the lean meat-sucrose diet described in Section 1. Plasma lipids were determined at various intervals after pancreatectomy, and when a drop below normal was definitely established, pancreas fractions were added to the diet and their feeding continued for the next 20 to 24 days. Plasma choline was determined on three separate occasions: (1) on the day that the feeding of the fraction was begun, (2) on the last day of their feeding period, and (3) 2 to 3 weeks after their addition to the diet had been discontinued.

The feeding of Fraction AR for approximately 3 weeks produced a significant rise in the level of plasma choline in all six dogs studied (Table III). The choline content of the plasma of Dog D502 rose from 34 to 62 mg. per 100 cc., whereas in Dog D562 the level of plasma choline was more than doubled by the feeding of Fraction AR. The higher levels of plasma choline produced by the feeding of Fraction AR were not maintained after its feeding was discontinued. The values observed 2 to 3 weeks after the feedings of Fraction AR were stopped were in close agreement with those observed at the start of the experiment.

The ingestion of as little as 60 mg. per day of Fraction 27C led to pronounced increases in the level of plasma choline. It was found earlier that 60 mg. per day of this fraction were also sufficient to maintain a normal fat content in the livers of completely depancreatized dogs for as long as 6 months (6).

*The results obtained with Fraction 27C leave no doubt that pancreas contains a substance highly active in choline metabolism.*

4. *The ingestion of pancreas fractions increases circulating choline in depancreatized dogs fed a low choline diet.* After pancreatectomy Dogs D561, D511A, D511B, D520A, and D520B were fed the meat-sucrose diet. When their plasma choline was reduced below normal, they were placed on the low choline diets described in Table IV. 2 weeks later 1 gm. of Fraction AR was added to their diets and this amount fed daily for the next 3 weeks. Plasma choline was determined on the following three occasions: (1) on the 14th day after the feeding of the low choline diet was begun, *i.e.* on the 1st day that Fraction AR was added to the diet; (2) 21 days later, *i.e.* on the last day that Fraction AR was fed; and (3) 3 weeks after Fraction AR had been excluded from the diet. The results of this experiment are recorded in Table IV.

TABLE IV

*Effect of Pancreas Fractions on Plasma Choline of Depancreatized Dogs Fed Diet Low in Choline*

Dog No.	Weight	Diet				Pancreas fraction				Plasma choline
		Ex- tracted casein	Sucrose	Lard	Days diet fed		Total days fed	Amount fed daily		
								gm. per meal	Type	
	kg.	gm. per meal	gm. per meal	gm. per meal				gm.		mg. per 100 cc.
D561†	7.4	35*	66		14	Begun		1.0	AR	42.2
	8.0	35	66		35	Stopped	21			65.5
	7.5	35	66		56					47.2
D511A†	7.5	35*	66		14	Begun		1.0	AR	51.5
	8.6	35	66		35	Stopped	21			63.3
	8.1	35	66		56					37.2
D511B‡	7.0	35*	66	10	14	Begun		1.0	AR	35.8
	7.5	35	66	10	35	Stopped	21			65.2
	7.2	35	66	10	56					47.2
D520A†	10.6	35*	66		14	Begun		1.0	AR	56.5
	11.0	35	66		35	Stopped	21			67.5
	10.7	35	66		56					48.0
D520B‡	10.5	35*	66	10	14	Begun		1.0	AR	50.1
	11.0	35	66	10	35	Stopped	21			67.5
	10.6	35	66	10	56					55.0
D509A§	11.0	40	50	10	14	Begun		100	27C	41.0
	10.0	40	50	10	35	Stopped	21			86.0
	9.6	40	50	10	56					33.3
D509B§	8.7	40	50	10	14	Begun		100	27C	33.3
	8.4	40	50	10	35	Stopped	21			67.5
	7.0	40	50	10	56					37.8
D512§	10.8	40	50	10	14	Begun		100	27C	41.3
	10.2	40	50	10	35	Stopped	21			57.2
	10.0	40	50	10	56					42.7

\* The casein was extracted for several days with increasing concentrations of methanol, the final extraction having been made with 90 per cent hot methanol for 1 day. It was free of choline.

† These dogs received once daily 5 cc. of Galen B, 2 gm. of Cowgill's salt mixture, and 4 gm. of bone ash.

‡ These dogs received once daily 5 cc. of Galen B, 3 cc. of cod liver oil, 2 gm. of Cowgill's salt mixture, and 4 gm. of bone ash.

§ These dogs received with each meal 2 gm. of bone ash, 3 cc. of cod liver oil, and 20 gm. of Cellu flour. Each dog received per kilo per day 100 γ of thiamine, 100 γ of riboflavin, 100 γ of pyridoxine, 2 mg. of niacin, 2 mg. of calcium pantothenate, and 2 mg. of inositol.

|| Vitamin-free Smaco casein was used.

Dogs D509 and D512 were fed the meat-sucrose diet for their first 73 and 62 days respectively after pancreatectomy. At the end of this period the values for plasma choline were below the preoperative. The subsequent treatment of these two dogs is recorded in Table IV. The values for plasma choline shown in Table IV were obtained (1) at the end of the first 2 weeks in which they were fed the low choline diet; (2) 21 days later, during which time they received daily the low choline diet supplemented with 100 mg. of pancreas Fraction 27C; (3) 21 days later, during which time they were fed the low choline diet without the pancreas fraction.

The lean meat-sucrose diet employed in the experiments described in Sections 1 to 3 was quite rich in choline. The quantity of meat fed each dog contained approximately 0.5 gm. of choline, an amount that is more than enough to account for the extra choline that appears in the plasma of the depancreatized dog in response to the ingestion of the antifatty liver factor of the pancreas. As judged by the results of Table IV, however, the antifatty liver factor of the pancreas increases the concentration of plasma choline even when the diets fed are quite low in choline.

With the exception of the rice bran concentrate (Galen B) the constituents in the diet fed to Dogs D561, D511A, D511B, D520A, and D520B were practically free of choline. The 5 cc. of Galen B fed each day contained about 60 mg. of choline. The amount of Fraction AR added to the diet daily could have contributed about 10 mg. of choline. Although the rise in plasma choline, which in these three dogs amounted to 15 to 30 mg. per 100 cc. of plasma, could have been derived from the small amounts of dietary choline, the extra choline that appeared in the plasma of Dogs D509 and D512 when 100 mg. of Fraction 27C were added to the diet could not have originated from dietary choline. These two dogs received a diet practically free of choline. The casein, Cellu flour, as well as the purified B vitamins, contained no measurable amounts of choline. Vitamins A and D were furnished in the form of cod liver oil; the 3 cc. fed daily to each dog contained less than 0.15 mg. of choline. The lard fed daily contained less than 0.5 mg. of choline. Thus, although each dog ingested less than 1 mg. of choline daily, the extra choline that appeared in plasma alone when 100 mg. of pancreas Fraction 27C were administered amounted to well over 200 mg.

#### DISCUSSION

Although it has been widely accepted that phospholipids serve to transport fat, their rôle as vehicles for the storage and transport of choline has not hitherto been stressed. There can be no doubt from the evidence presented in the preceding paper that practically all the choline contained in plasma is in firm organic combination as phospholipids. This observa-

tion in conjunction with a unique characteristic of plasma phospholipids, namely that they are all of the choline-containing type, would appear to lend support to the view that plasma phospholipids are intimately connected with the physiological activity of *circulating* choline.

The results of the present investigation demonstrate that the complete excision of the pancreas or, more specifically, the deprivation of the external secretion of the pancreas profoundly affects choline metabolism. In the dog deprived of its pancreatic juice, there occurs a reduction in the concentration of circulating or plasma choline. This decrease in plasma choline occurred even though the dogs were fed diets that were not deficient in choline or its precursor, methionine.

It is evident, therefore, that a relation exists between the level of plasma choline and the external secretion of the pancreas. This is further borne out by the isolation of a pancreas fraction that is active in maintaining the level of circulating choline when added to the diet of the dog deprived of the external secretion of its pancreas. The administration of as little as 60 mg. daily of such a fraction was found sufficient to raise the concentration of plasma choline to normal.

While the mechanism by which the pancreas, through its external secretion, exercises a controlling influence on the level of circulating choline remains at present unexplained, it is of interest to note here that the action of pancreas fractions in increasing the concentration of plasma choline is not dependent upon the presence of choline as such in the diet. The extra choline that appears in the plasma of depancreatized dogs receiving pancreas fractions must therefore result from either a shift of choline from the tissues to the plasma or the synthesis of choline from its precursors.

#### SUMMARY

1. The concentration of plasma choline is depressed in dogs deprived of the external secretion of the pancreas and in completely depancreatized dogs maintained with insulin. This fall in plasma choline is associated with the development of fatty livers.

2. A principle highly active in choline metabolism can be isolated from the pancreas. The daily administration of as little as 60 mg. of a pancreas fraction (No. 27C) is sufficient to prevent the fall in plasma choline in depancreatized dogs, as well as to raise the level in those in which the level of plasma choline had been permitted to fall. This fraction of pancreas also prevents fatty livers in depancreatized dogs maintained with insulin.

3. This action of pancreas fractions in increasing the concentration of plasma choline is *not* dependent upon the presence of choline as such in the diet.

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# INTERACTION BETWEEN PROTEINS AND SYNTHETIC DETERGENTS

## III. MOLECULAR-KINETIC STUDIES OF SERUM ALBUMIN-SODIUM DODECYL SULFATE MIXTURES\*

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Attempts have been made to explain the action of certain protein-denaturing agents, such as organic solvents, urea, guanidine halides, and salicylates, in terms of (a) physical solvent effects (*cf.* (4)) or (b) unspecified intermediary combination between denaturant and protein (*cf.* (5)). Recent work has shown that the denaturing effect of guanidine hydrochloride, one of the most powerful denaturants hitherto known, is equaled by that of synthetic detergents and that the latter are effective in considerably lower concentrations (6-8).

Chemical and electrophoretic measurements have revealed the formation of stoichiometric complexes of horse serum albumin and sodium dodecyl sulfate (SDS), presumably involving the ionized basic groups of the protein molecule (1, 2, 9). This view is further supported by viscosity and diffusion measurements on serum albumin-SDS mixtures presented in this paper. It is proposed that combination of SDS with specific side chain groups of the protein is an important step in denaturation.

### EXPERIMENTAL

*Materials and Methods*—The materials used in this study were five times crystallized horse serum albumin,<sup>1</sup> containing 0.1 per cent carbohydrate, prepared by the method already described (1), and purified sodium dodecyl sulfate.<sup>2</sup> Both substances were homogeneous in electrophoresis (2). Stock solutions were prepared by dissolving in buffer weighed amounts of lyophilized salt-free albumin or of detergent. The composition of the phosphate-NaCl buffer used in most of this work was 0.01 M

\* The first paper in this series described the effect of detergents on the precipitation of proteins (1), Paper II, the electrophoretic analysis of serum albumin-sodium dodecyl sulfate mixtures (2). A preliminary account of this work has been given (3).

<sup>1</sup> We are indebted to the Lederle Laboratories, Inc., Pearl River, New York, for a supply of horse serum.

<sup>2</sup> A gift of Dr. S. Lenher of the Fine Chemicals Division of E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware. For the chemical analysis see the first paper (1).

$\text{KH}_2\text{PO}_4$ -0.01 M  $\text{Na}_2\text{HPO}_4$ -0.15 M NaCl,  $\mu$  approximately 0.2, pH 6.7 (determined with the glass electrode). Other buffers used in the preliminary investigation were of the following composition: 0.05 M sodium acetate-0.05 M NaCl,  $\mu = 0.1$ , pH 4.2; 0.02 M sodium acetate-0.2 M NaCl,  $\mu$  approximately 0.2, pH 5.0; and 0.02 M total phosphate-0.15 M NaCl,  $\mu$  approximately 0.2, pH 7.7.

*Viscosity* measurements were carried out at  $25^\circ \pm 0.01^\circ$  with the modified Ostwald viscometers previously described (10).

*Density* measurements were made at  $25^\circ$  in 5 cc. capped pycnometers.

*Diffusion* measurements were performed with the refractometric scale method at  $25^\circ \pm 0.003^\circ$ . The apparatus and methods used in the calculation of diffusion constants have been described in detail in a recent review (11).

The *protein concentration* of stock solutions and of their dilutions was determined from the weight of samples after correction for moisture content, and confirmed by Kjeldahl nitrogen analysis.

*Preliminary Viscosity Measurements*—Preliminary viscosity measurements were undertaken<sup>3</sup> at pH 4.2 and 7.7 on mixtures of horse serum albumin with (a) a commercial anionic detergent,<sup>4</sup> (b) a commercial cationic detergent,<sup>5</sup> and (c) purified SDS. At both pH values all three detergents produced large increases in the relative viscosity of the protein. However, further investigation was restricted to purified SDS.

Viscosity measurements made 30 minutes after mixing albumin and detergent, and repeated at intervals up to 48 hours, gave values that were the same within the experimental error. As a routine precaution, all mixtures were allowed to stand overnight at room temperature.

*Constant Detergent Concentration*—As a corollary to the study of the effect of detergent concentration on protein precipitation, measurements were made of the relative viscosity of serum albumin at different dilutions in solutions of constant detergent concentration. To avert precipitation, measurements at pH 4.2 were limited to the region of detergent excess.

In Fig. 1, the viscosity<sup>6</sup> of the protein relative to that of the solvent (considered to be buffer plus SDS) is plotted against the protein concentration in weight per cent. It is apparent that low molar concentrations

<sup>3</sup> We are indebted to Dr. John O. Erickson for these preliminary measurements.

<sup>4</sup> Nacconol N. R. S. F., a sodium alkyl benzenesulfonate mixture, National Aniline Division, Allied Chemical and Dye Corporation.

<sup>5</sup> Emulsept, N (higher acyl esters of colaminoformylmethyl) pyridinium chloride, The Emulsol Corporation.

<sup>6</sup> The relative viscosity,  $\eta/\eta_0$ , is the viscosity of the solution divided by that of the pure solvent. The term  $(\eta/\eta_0 - 1)$  is the specific viscosity  $\eta_{sp}$ , and  $\eta_{sp}/c$  is the viscosity increment (12), where  $c$  is the volume (or weight) concentration of the solute. The intercept of the plot of  $\eta_{sp}/c$  against  $c$  is the intrinsic viscosity (cf. (13)).

of detergent give rise to a viscosity increase comparable to that produced by high concentrations of urea or guanidine hydrochloride. Thus, 8 M urea increases the intrinsic viscosity<sup>6</sup> of serum albumin from 4.3 to about 24 (14), 0.035 M SDS (1 per cent) to 18, and 0.17 M SDS (5 per cent) to 26. Moreover, 0.28 M SDS (8 per cent) has about the same effect (29.5) as

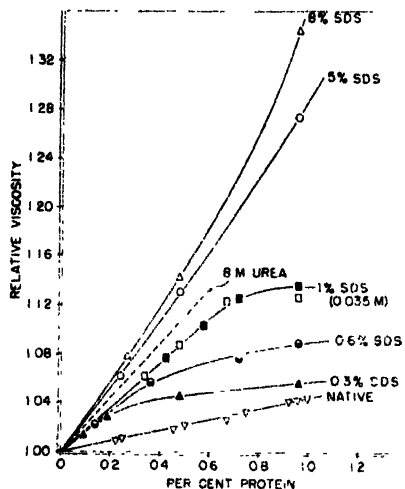


FIG. 1

FIG. 1. Relative viscosity of horse serum albumin in sodium dodecyl sulfate (SDS) solutions of constant concentration. The viscosity of the protein relative to that of the solvent (SDS in buffer) is plotted against the protein concentration in weight per cent. In order of decreasing slope the curves refer, respectively, to serum albumin in 8 per cent SDS, pH 4.2; in 5 per cent SDS, pH 4.2; in 1 per cent SDS, pH 4.2 ( $\square$ ), and pH 6.7 ( $\blacksquare$ ); in 0.6 per cent SDS, pH 6.7; in 0.3 per cent SDS, pH 6.7; and to native serum albumin in buffers of pH 4.2, 5.0, and 6.7. The dotted line serves as a comparison to the relative viscosity of denatured horse serum albumin in 8 M urea.

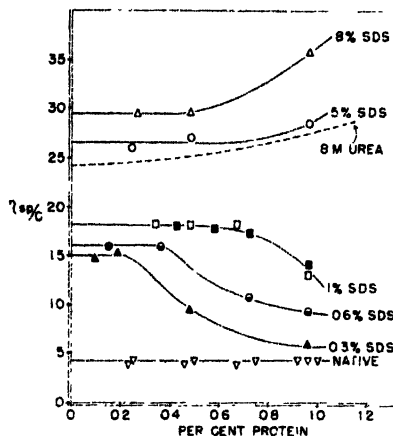


FIG. 2

FIG. 2. Read from top to bottom the curves refer, respectively, to serum albumin in 8 per cent sodium dodecyl sulfate (SDS), pH 4.2; in 5 per cent SDS, pH 4.2; in 1 per cent SDS, pH 4.2 ( $\square$ ), and pH 6.7 ( $\blacksquare$ ); in 0.6 per cent SDS, pH 6.7; in 0.3 per cent SDS, pH 6.7; and to native serum albumin in buffers of pH 4.2, 5.0, and 6.7. The dotted line represents denatured horse serum albumin in 8 M urea.

8 M guanidine hydrochloride (31.5) which gives the highest value thus far observed (14).

The close agreement between the values obtained in solutions containing 1 per cent SDS at pH 4.2 and 6.7 (Fig. 1) illustrates the fact that the viscosity increase is independent of pH.

Whereas in dilute solutions the viscosity increment<sup>6</sup> is usually independent of protein concentration, as shown in Fig. 2 for native albumin,

in relatively *high* concentrations of SDS, as in 8 M urea or guanidine hydrochloride (14),  $\eta_{sp}/c$  increases with protein concentration. In contrast, in relatively *low* detergent concentrations (1 per cent or less),  $\eta_{sp}/c$  decreases with increasing protein concentration (cf. Fig. 2) and tends to approach the value observed for the native protein.

*Dependence of Viscosity on Detergent to Protein Weight Ratio*—The data presented in Fig. 2 suggest that in low detergent concentrations, at least over a certain range, the viscosity increment depends on the detergent to protein ratio. As a test of this hypothesis additional viscosity measurements have been made and all of the data have been plotted in Fig. 3, the viscosity increment being given as a function of the detergent to pro-

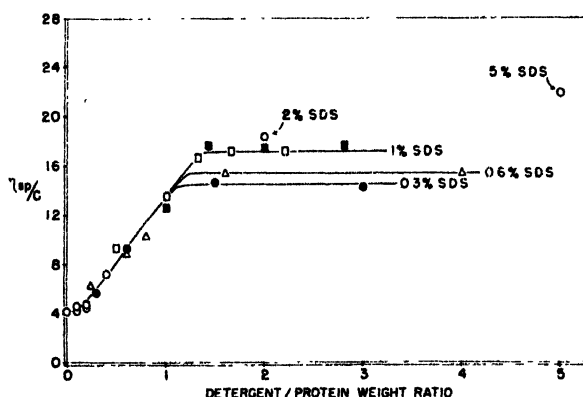


FIG. 3. Viscosity increment,  $\eta_{sp}/c$ , of horse serum albumin in sodium dodecyl sulfate (SDS) solutions at pH 6.7, plotted against the detergent to protein weight ratio. Reading from top to bottom the curves refer, respectively, to protein in 1 ( $\square$ ), 0.6 ( $\triangle$ ), and 0.3 ( $\bullet$ ) per cent SDS. All other data at pH 6.7 are represented by clear circles. Solid squares indicate the viscosity increment of serum albumin at pH 4.2.

tein weight ratio. Three regions may be recognized for each of which a correlation with precipitation and electrophoresis data obtains: (1) Initially, the viscosity increment is nearly independent of detergent to protein weight ratio, up to a value of approximately 0.2. This ratio corresponds to the composition of the electrophoretic complex  $AD_n$  at pH 6.7, and coincides with the minimal ratio required for complete precipitation of the protein at pH 4.5. (2) The segment of the curve in which the viscosity increment increases linearly with detergent to protein ratio corresponds to the formation of  $AD_{2n}$  and higher, electrophoretically homogeneous complexes. (3) Beyond the point at which the viscosity increment no longer depends on detergent to protein weight ratio (approximately 1),

free detergent first makes its appearance in electrophoresis, the mixtures being completely soluble at pH 4.5. In this region, the viscosity increment appears to be dependent on the absolute detergent concentration.

The dependence of the viscosity increment on detergent to protein weight ratio indicates that up to a ratio of approximately 1, detergent is bound by the protein, a conclusion in accord with electrophoretic measurements (2). However, electrophoresis also revealed that, depending on the detergent to protein ratio, one or more components may be present. Therefore, determinations of the viscosity increment at infinite dilution (intrinsic viscosity<sup>6</sup>) were limited to mixtures corresponding in composition to electrophoretically homogeneous complexes.

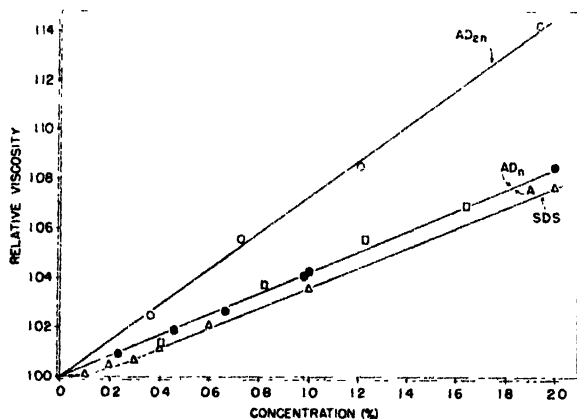


FIG. 4. Relative viscosity of serum albumin,  $A$  ( $\bullet$ ), and sodium dodecyl sulfate, SDS ( $\Delta$ ), and of the stoichiometric complexes  $AD_n$  ( $\square$ ) and  $AD_{2n}$  ( $\circ$ ) at pH 6.7, plotted against the total weight concentration of solute (albumin plus detergent). For the composition of the complexes see the text. Extrapolation to infinite dilution of SDS yields a deviation from linearity indicated by the dotted portion of the curve and discussed in the text.

*Molecular-Kinetic Studies of Complexes  $AD_n$  and  $AD_{2n}$* —Solutions of serum albumin and SDS were made up in phosphate buffer, pH 6.7, the mixing ratio of the components corresponding, respectively, to complexes  $AD_n$  (0.225 gm. of SDS per gm. of albumin) and  $AD_{2n}$  (0.45 gm. of SDS per gm. of albumin). Viscosity measurements were made on successive dilutions of these two stock solutions with buffer, thereby maintaining constant relative composition.

The relative viscosities of solutions of these complexes, as well as of native serum albumin and of pure SDS, are plotted in Fig. 4 against the gm. per cent concentration of the solute. Since the detergent is assumed to be completely bound by the protein, the viscosity was calculated relative to that of the buffer only.

It is to be noted that the viscosity of pure SDS<sup>7</sup> is almost the same as that of pure serum albumin. In the absence of interaction other than that restricted wholly to volume additivity, no significant viscosity increase would be anticipated when the relative viscosity of the mixtures is plotted against the total solute concentration. This, indeed, is the case for  $AD_n$ , and confirms the previous conclusion that up to a detergent to protein ratio of about 0.2 no significant viscosity increase occurs. However, this is not the case for the second complex,  $AD_{2n}$ , which shows a significant viscosity increase, attributable to a corresponding change in molecular dimensions.

From the intrinsic viscosity, apparent molecular shapes (hydration being disregarded) of the pure components and of the two complexes were calculated in the manner already described (10), assuming the molecules to approximate prolate ellipsoids of revolution. The data are expressed in terms of axial ratios,  $b/a$ , and are given in Table I, together with the

TABLE I  
*Molecular-Kinetic Constants of Protein-Detergent Complexes*

Material	$\left(\frac{\eta_{sp}}{c}\right)_{c \rightarrow 0}$	$\frac{b}{a}$	$\left(\frac{b}{a}\right)_h$	$\left(\frac{f}{f_0}\right)_\eta$	$D_{25}'$ <small><math>10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}</math></small>	Mol. wt.
Serum albumin.....	4.20	4.8	3.3	1.24	7.0*	70,000*
Sodium dodecyl sulfate..	4.20	4.2		1.20	10.3†	22,000
$AD_n$ .....	4.30	4.8	3.3	1.24	6.9	76,000
$AD_{2n}$ .....	7.25	7.4	5.6	1.40	5.5	98,000

For an explanation of the symbols see the text.

\* Data of Neurath *et al.* (14).

† Diffusion data of Hakala (16), corrected to 25°.

corresponding dissymmetry constants,  $f/f_0$ , and shape values corrected for 33 per cent hydration,  $(b/a)_h$ .

The data indicate that, while native serum albumin, pure SDS, and the first complex,  $AD_n$  all have about the same molecular shapes, the second complex,  $AD_{2n}$ , is about twice as elongated.

*Diffusion* constants of native horse serum albumin (11) and of SDS (16) have been taken from the literature, the latter value representing the average of an extensive series of measurements. Diffusion constants of  $AD_n$  and  $AD_{2n}$  were measured on the solutions used for viscosity studies.

<sup>7</sup> The apparent break in the viscosity curve for pure SDS (Fig. 4) may correspond to the onset of ionic micelle formation. For a discussion of this phenomenon see (15).

Calculations were made by five different methods as indicated in Table II in which the results are recorded in detail.

The mixtures of serum albumin and SDS represented by complexes  $AD_n$  and  $AD_{2n}$  appear to be homogeneous in diffusion, as indicated by the agreement of the values computed by the several methods of calculation (11). This observation is also in accord with the fact that solutions of these complexes are essentially homogeneous in electrophoresis (2).

The data in Table I reveal that the diffusion constant of  $AD_n$  and particularly that of  $AD_{2n}$  are lower than those of the individual constituents.

TABLE II  
*Diffusion Constants of Serum Albumin-Sodium Dodecyl Sulfate Complexes\**

	Time	$D_1$	$D_2$	$D_3$	$D_4$	$D_5$
	sec	$10^{-7}$	$10^{-7}$	$10^{-7}$	$10^{-7}$	$10^{-7}$
$AD_n$ (0.67% horse serum albumin-0.15% SDS)†	78,960	6.7	6.7	6.9	6.9	
	100,920	6.6	6.6	6.8	7.0	$6.7 \pm 0.2$
	123,960	6.6	6.6	6.8	7.0	
Average		$6.75 \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$				
$D'$		$6.9 \times 10^{-7} \text{ " " " " "}$				
$AD_{2n}$ (0.50% horse serum albumin-0.225% SDS)†	81,600	5.3	5.4	5.6	5.9	
	103,020	5.2	5.3	5.3	5.5	$5.45 \pm 0.1$
	127,260	5.3	5.2	5.5	5.7	
Average		$5.45 \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$				
$D'$		$5.5 \times 10^{-7} \text{ " " " " "}$				

\*  $D$  denotes the diffusion constant at  $25^\circ$  in sq. cm. per second;  $D_1$ ,  $D_2$ ,  $D_3$ ,  $D_4$ , and  $D_5$  refer, respectively, to the diffusion constant calculated by the maximum height, maximum height-area (unsquared), maximum height-area (squared), standard deviation, and successive analysis methods.  $D'$  is the average diffusion constant corrected for solvent viscosity (11).

† Buffer composition, 0.01 M  $\text{KH}_2\text{PO}_4$ -0.01 M  $\text{Na}_2\text{HPO}_4$ -0.15 M NaCl, pH 6.7, ionic strength approximately 0.2.

This is in qualitative agreement with the observations of Lundgren *et al.* for egg albumin-sodium alkyl benzenesulfonate mixtures (17), whereas Miller and Andersson (18) have observed intermediate values of the diffusion constant for the complex formed between insulin and Duponol.

*Molecular weights* were calculated from the values of the dissymmetry constants and diffusion constants and are given in the last column of Table I. It may be noted that the molecular weight of the detergent micelle is about 20,000 (somewhat higher than previously reported for Duponol (18)) and that the molecular weights of the complexes  $AD_n$  and  $AD_{2n}$  exceed that of either albumin or detergent. The molecular weight



of the complex,  $M_{12}$ , may also be calculated from the combining weight ratio,  $s$ , and the molecular weight of native albumin,  $M_1$ , with the equation (19),

$$M_{12} = (s + 1)M_1$$

where  $s$  represents gm. of SDS combined per gm. of protein (data obtained by electrophoresis (2)). The corresponding values calculated for  $AD_n$  and  $AD_{2n}$  are, respectively, 85,600 and 101,500.

It is noteworthy that in the formation of these two complexes at pH 6.7 neither aggregation (as manifested by precipitation below pH 4.8 (1)) nor dissociation (as observed for insulin (18)) occurs. It may be concluded, therefore, that detergent reacts with the albumin to form molecularly and electrophoretically homogeneous complexes of definite composition.<sup>8</sup>

#### DISCUSSION

The behavior of serum albumin-SDS mixtures with respect to precipitation, viscosity, and electrophoresis may be fully accounted for by postulating the formation of discrete stoichiometric complexes. Though precipitation is restricted to the pH range in which the components carry charges of opposite sign, complex formation, as well as denaturation, is independent of pH over a wide region. Viscosity measurements on solutions of the homogeneous complexes,  $AD_n$  and  $AD_{2n}$ , indicate the intrinsic viscosity of the first complex to be nearly the same as that of the native albumin, whereas that of the second is considerably higher. The formation of electrophoretic complexes higher than  $AD_{2n}$  coincides with further increase in the viscosity of the mixtures.

In previous work, the increase in intrinsic viscosity of proteins upon denaturation by various means has been considered to be due primarily to an increase in asymmetry of the protein molecules rather than to increased solvation<sup>9</sup> (14, 20-22). The same point of view has been adopted

<sup>8</sup> In preliminary measurements of the sedimentation of horse serum albumin in Duponol (a mixture of homologous sodium alkyl sulfates, approximating SDS in mean molecular weight) Miller and Andersson (18) noted that 1 per cent serum albumin in 0.5 per cent Duponol (a weight ratio closely corresponding to that found for  $AD_{2n}$ ) "yielded on ultracentrifugation a single, fairly symmetrical boundary. . . The areas under the curves indicated the presence of the Duponol as well as the serum albumin." The fact that a slightly lowered sedimentation constant was obtained indicated a possible dissociation of serum albumin analogous to that observed with insulin. However, with their sedimentation constant and the diffusion constant observed by us for  $AD_{2n}$  (corrected to 20°), an apparent molecular weight of 90,000 may be calculated.

<sup>9</sup> If the increase in intrinsic viscosity of the protein were to be ascribed fully to combination with the denaturant, the amount combined may be calculated from the relation

in this paper. While in the present instance unsymmetrical adsorption of detergent anions by the protein might conceivably account for the increase in intrinsic viscosity of the complexes, the occurrence of other changes, recognized as criteria of denaturation, militates against this hypothesis. For example, the full complement of sulfhydryl groups of egg albumin is liberated both by 8 M guanidine hydrochloride (23) and by relatively low concentrations of alkyl sulfates (7). Moreover, the viscosity increase of egg albumin in concentrated detergent solutions is accompanied by the development of positive flow birefringence indicative of rod-shaped particles and by visible fiber formation upon extrusion into coagulating solutions (24). The x-ray diffraction patterns of regenerated fibers are similar to those obtained with proteins denatured by other means (20, 25).

While the exact structure of the complexes and the exact nature of the processes involved in their formation remain to be elucidated, certain deductions may be made from the data presented herein and in the preceding papers of this series (1, 2). The stoichiometric combination between detergent and proteins in the formation of both  $AD_n$  and  $AD_{2n}$  presumably is due to ionic interaction. The greater reactivity of the groups which combine in the first complex may be ascribed to a difference in dissociation constant, spatial position, or structural function. It is stated that slightly more than one-half of the cationic groups of serum albumin are strongly basic (62 guanidinium and  $\epsilon$ -amino (26)) and it has been found that nearly the same number (about 55) are combined in the first complex (2). With other proteins, *e.g.* egg albumin (27) and  $\beta$ -lactoglobulin,<sup>10</sup> the formation of two discrete complexes with SDS has also been observed. However, though the composition of the higher complex may be related in both instances to the total acid-binding capacity of the protein, a specific assignment of strongly or more weakly basic protein groups to either complex does not seem warranted on the basis of the available analytical data.

$$\frac{(\eta_{sp}/c)_H}{(\eta_{sp}/c)_A} = \frac{V_H}{V_A}$$

where the subscripts *H* and *A* refer to the intrinsic viscosity in the presence of the denaturant and in aqueous buffer, respectively.  $V_H$  and  $V_A$  denote the respective specific hydrodynamic volumes of the solvated and anhydrous protein. In this connection it should be noted that on previous occasions (14, 20) the amount of urea that would have to be bound per gm. of serum albumin to explain the viscosity increase observed in 8 M solutions has been given as 2.7 gm., whereas the correct value is 4.2 gm. Accordingly, it is in a 4 per cent protein solution that about one-third of the urea molecules would have to be bound and in a 10 per cent solution all of them (*cf.* (20)).

<sup>10</sup> Unpublished experiments.

As an alternative, it may be postulated that the groups combined in the second complex differ from the remainder in accessibility, either as a result of steric arrangement or because they are involved in side chain bond formation. Both hypotheses are compatible with the fact that all of the basic groups are titratable with acids, the former, because these groups may be accessible to hydrogen ions but not to large detergent anions, the latter, because the bonds are broken during titration. Regardless of whether some of the ionized protein groups occupy positions within the molecule (as postulated by a proposed structure for an egg albumin-detergent complex<sup>11</sup> (28)), they may be involved in side chain bond formation.

Recent discussions of protein structure have emphasized the structure-determining rôle of side chain interaction (29-31) and the hypothesis of electrostatic bonds between some of the ionized protein groups is in accordance with these views. Breaking of these bonds by detergents or hydrogen bond-forming compounds (*e.g.*, urea, salicylate, guanidine hydrochloride) will lead to side chain separation followed by disorientation of the main chains, as evidenced by viscosity increase and other changes observed in denaturation. Of course, the breaking of only a few of these bonds may be critical, as in the case of pepsin and hemoglobin (*cf.* (20)).

The second complex,  $AD_{2n}$ , represents a well defined species characteristic of a certain degree of denaturation. The formation of the higher complexes may be ascribed to the non-polar adsorption of additional detergent by this complex, either by non-polar protein groups or by the detergent already bound.<sup>12</sup> The further increase in intrinsic viscosity observed in this region coincides with the dissolution of the precipitate at pH 4.5 (*cf.* (1, 32)). Further disorientation of the protein occurs in this range as manifested by phenomena usually observed with a high degree of denaturation (see above (24)).

The results described in this series of papers have led to a more detailed description of the process of denaturation of proteins by synthetic detergents. This explanation may equally apply to denaturation by urea, salicylate, or guanidine hydrochloride. The considerably higher concentrations required for the latter agents to exert their full effects may be ascribed to their lower affinity for the protein groups, rather than to a mere solvent effect.

<sup>11</sup> The primary assumption made by Palmer (28) in his discussion of the structure of the egg albumin-detergent complex, namely that the molecules are "held together by non-specific, non-polar forces," is not in accord with the conclusions arrived at in this series of papers (1, 2).

<sup>12</sup> It is unlikely that  $AD_{2n}$  results from non-polar adsorption of a second layer of detergent to  $AD_n$ , with hydrophilic groups exposed, because (1) both  $AD_n$  and  $AD_{2n}$  are insoluble below pH 4.8, and (2)  $AD_{2n}$  but not  $AD_n$  exhibits a large viscosity increase over the native albumin.

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#### SUMMARY

In continuation of previous studies of the interaction between proteins and synthetic detergents, the viscosity of mixtures of horse serum albumin and sodium dodecyl sulfate has been investigated.

At constant detergent concentration, the viscosity increment of the protein is independent of pH but varies with the detergent to protein ratio. Apparent viscosity anomalies observed with low detergent to protein ratios have been attributed to the formation of stoichiometric complexes whose existence had already been demonstrated by electrophoretic analysis.

The molecular-kinetic properties of the discrete complexes,  $AD_n$  and  $AD_{2n}$ , have been determined by diffusion and viscosity measurements, and compared to those of the pure components. While formation of the first complex occurs without detectable changes in molecular shape, formation of the second complex involves an increase in the molecular asymmetry of the protein. More extensive denaturation of the protein occurs as higher complexes of variable composition are formed.

The structure of the complexes and the mode of denaturation have been interpreted in terms of stoichiometric binding of detergent anions by cationic protein groups with resultant rupture of structure-determining protein side chain bonds. It is suggested that other denaturants of lower affinity for proteins act in a similar manner.

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# GROWTH REQUIREMENTS OF A PURINE-DEFICIENT STRAIN OF *NEUROSPORA*\*

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Among the mutant strains of *Neurospora crassa* isolated by Beadle and Tatum (1) was one, strain 3254, which although unable to grow on the simple medium of salts, sugar, and biotin grew in the presence of yeast extract. Preliminary experiments showed that this strain did not respond to the known vitamin and amino acid mixtures, but that adenine was an effective growth supplement. The rate of growth on adenine as on yeast extract itself, however, failed to reach the value found for the normal wild type regardless of the concentration used. In an attempt to find the constituent which would provide for normal growth and to compare their growth-promoting activities, various purines and related compounds have been studied. Of the compounds examined a cozymase preparation gave the highest maximum rate of growth, a value about 75 per cent that of the wild strain. The other active compounds, adenine, hypoxanthine, oxyadenine, adenosine, adenosine-3-phosphate, and yeast nucleic acid, as well as extracts of several natural products including liver, malt, and *Neurospora*, gave comparable maximum growth rates equal to 60 to 70 per cent that of the wild type. As these maximum rates were in no case equal to that of the wild strain, it appears that, while this mutant is deficient in adenine or the closely related purines, there are other genetic factors which result in a reduced rate of growth.

A comparison of the growth-promoting efficiency of the different active compounds at concentrations less than those required for maximum growth showed that the rate was dependent on the amount of adenine present whether free or combined as adenosine or adenosine-3-phosphate (yeast adenylic acid). Yeast nucleic acid as the sodium or ammonium salt provided rates roughly comparable to that expected from the adenine content required by the tetranucleotide theory. The compounds most closely related structurally to adenine, hypoxanthine and oxyadenine, proved as effective as the former in equivalent concentrations. While no specificity was found in these cases, the other purines including guanine, xanthine,

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caffeine, and uric acid, and allantoin and uracil were completely inactive. The present paper presents the results of these experiments.

#### EXPERIMENTAL

*Measurement of Growth*—Two methods have been used previously for measuring the growth of *Neurospora*; namely, the rate of progression of the mycelium on an agar medium in horizontal tubes (1, 2) and the dry weight of mycelium produced in liquid culture (3). While the latter has been found more satisfactory for assay purposes (3-5), it has been observed that some mutant strains which produce maximum growth in liquid culture (an amount comparable to that produced by the original wild strain) fail to do so when grown on an agar medium. The reason for this difference is not entirely clear, but it appears that such strains respond to non-specific substances present in the liquid medium. Because the present mutant, strain 3254, responded in this manner when grown in the presence of yeast extract, its growth response was measured by the former method at 25°. The composition of the basal medium and the method of preparation and inoculation of the growth tubes have been previously described (3, 6). The position of the mycelial frontier was marked for the first time 24 hours after inoculation, which allowed sufficient time for the mycelial frontier to have become well established. The tubes were marked at 24 hour intervals for a period of 5 or 6 days, and the average rate in mm. per hour was calculated for each day. The stock cultures were maintained on agar slants containing the basal medium supplemented with 1 mg. of adenine sulfate per 10 ml. Transfers were made about every 3 weeks.

*Materials*—The purified compounds and extracts of natural products, in most instances, were obtained from commercial sources as follows: adenine, xanthine, uracil, and yeast nucleic acid, Eastman Kodak Company; adenosine and yeast adenylic acid, A. D. Mackay Company; yeast extract, Difco Laboratories; solubilized liver, Wilson and Company; malt extract, Peerless Yeast Company.

The following compounds were prepared in crystalline form by well known procedures: hypoxanthine ((7) p. 121), guanine ((7) p. 110), guanosine ((7) p. 163), trisodium guanylate (8), and allantoin (9).

Oxyadenine was kindly provided by Dr. Mary V. Buell and the sample of cozymase by Dr. Paul D. Boyer. The latter sample had been prepared by Merck and Company, Inc., and was stated to be 60 per cent pure coenzyme I.

The *Neurospora* extract was made by grinding a weighed amount of the dried wild type mycelia with the aid of a small amount of Hyflo Super-Cel and water in a mortar. Insoluble material was removed by centrifugation, and the aqueous extract was lyophilized.

*Results of Growth Experiments*—The growth characteristics of strain 3254 have been somewhat different from those that have been found either with the wild strain or with the pyrimidine-deficient mutants (6). With the latter, maximum growth rates of about 4.2 mm. per hour are established from 2 to 3 days after inoculation. In the case of strain 3254, however, the maximum value reached was only 3.0 mm. per hour regardless of the type or amount of supplement present in the basal medium. Similarly the time required to reach the maximum, when an optimum concentration of supplement was used, was increased to about 4 to 5 days. With slightly smaller amounts of supplement the same maximum rate was achieved but the time required was 5 or 6 days. With still smaller amounts of supplement the rate increased progressively over the first 4 days and reached a fairly constant rate, less than maximum after the 5th day. In the results presented below the maximum rates were arbitrarily chosen as those obtained during the 6th day.

Values for the various pure compounds and crude extracts and the approximate concentrations at which half maximum and maximum rates were obtained are given in Table I. It may be seen that, whereas rates of about 4.2 mm. per hour were obtained for the wild strain, the maximum rate found for strain 3254 on adenine was about 2.6 mm. per hour and that similar values were found for equivalent concentrations of oxyadenine, hypoxanthine, adenosine, and adenosine-3-phosphate. The cozymase preparation provided a slightly higher maximum rate, but the amount required indicated that its activity was dependent on the amount of adenine present in combined form. The results with yeast ribonucleic acid should be interpreted in a qualitative rather than quantitative sense. The concentrations are given in terms of samples prepared as the sodium and ammonium salts without regard to their phosphorus or moisture contents. The data show that adenine in these compounds is available for the growth of strain 3254 in the approximate concentrations expected.

The results with the various crude extracts suggest that either free or combined adenine or the closely related purines, oxyadenine or hypoxanthine, are present in varying concentrations in the preparations mentioned. The fact that none of the extracts including that from *Neurospora* itself produced a rate of growth comparable to that of the wild strain shows that the deficiency is not due entirely to the absence of a simple cell constituent, which would be expected to be present in aqueous extracts of ground *Neurospora*. It is possible that blockage of the mechanism for the *in vivo* synthesis of adenine or the related purines in strain 3254 leads either to the production of intermediates which inhibit growth or that adenine is not as readily absorbed or utilized for growth as are the various other compounds which produce normal growth in other deficient strains under similar conditions.



TABLE I

*Maximum Rates of Growth of Mutant 3254 on Various Supplements and Approximate Concentrations at Which Half Maximum and Maximum Rates Were Found*

Supplement	Concentration in medium		Rate of growth*
	For half maximum rates	For maximum rates	
	mg. per 10 ml.	mg. per 10 ml.	
Adenine sulfate, $(C_5H_5N_5)_2 \cdot H_2SO_4 \cdot 2H_2O$ .	0.1	0.5	2.6, 2.4, 2.5
Oxyadenine hydrochloride, $C_5H_5ON_5 \cdot HCl \cdot 2H_2O$ . . . . .	0.1	0.5	2.5, 2.7
Hypoxanthine, $C_5H_4ON_4$ . . . . .	0.1	1.0	2.6, 2.3
Adenosine, $C_{10}H_{13}N_5O_4 \cdot \frac{1}{2}H_2O$ . . . . .	0.15	0.7	2.5, 2.8
Adenosine-3-phosphate, $C_{10}H_{14}N_5O_7P \cdot 2H_2O$ . . . . .	0.3	0.9	2.7, 2.8
Yeast ribonucleic acid (neutral sodium salt) . . . . .	2.2	8.9	2.4
Yeast ribonucleic acid (neutral ammonium salt) . . . . .	2.2	8.9	1.9
Cozymase (60% pure) . . . . .	1.6	8.0	3.0
Yeast extract (Difco) . . . . .	16	40	2.1, 2.1
Liver extract (Wilson's liver fraction I) . . . . .	12	60	2.4, 2.6
Malt extract (Peerless) . . . . .	200	260	1.4
<i>Neurospora</i> extract . . . . .	50	100	2.8, 2.9

\* Each value represents the maximum rate found in a different experiment. Wild type controls on the basal medium alone grew at an average rate of 4.2 mm. per hour with the exception of the experiments on hypoxanthine and ribonucleic acid. In these cases, presumably owing to the presence of inhibiting substances in the agar, the wild type rate on the basal medium was 3.6 mm. per hour.

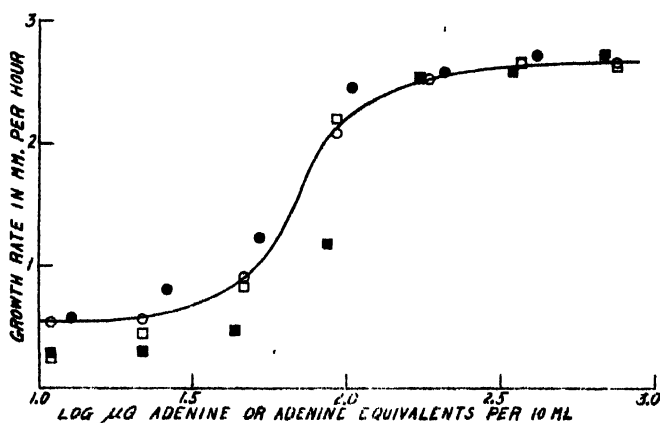


FIG. 1. Rates of growth of mutant strain 3254 as a function of concentration. ○ adenine, ● oxyadenine, □ adenosine, and ■ adenosine-3-phosphate.

The rates of growth on adenine, oxyadenine, adenosine, and adenosine-3-phosphate as a function of the logarithm of the concentration expressed as adenine or adenine equivalents in micrograms per 10 ml. are plotted in Fig. 1. The growth curves for this mutant are comparable to those published for other deficient *Neurospora* mutants with the exception of the lower maxima. The values plotted are rates obtained during the 6th day in an experiment carried out concurrently on the four compounds with the same agar and the same inoculum. It may be seen, within experimental error, that adenine, oxyadenine, and adenosine give the same growth curve when their concentrations are expressed in terms of adenine equivalents. Adenosine-3-phosphate gave a similar maximum but proved slightly less efficient at concentrations which failed to support maximum growth. Other experiments carried out at other times with the same supplements gave similar results. The growth curve obtained on hypoxanthine showed this compound to be less efficiently utilized for growth than adenine, but the results are less satisfactory because the agar available at the time contained some inhibiting material. The growth curve found with the cozymase preparation was similar to that found for adenylic acid, but the results are uncertain because no information was available as to whether or not the impurities known to be present contained adenine nucleosides or nucleotides.

The various compounds which proved inactive were tested at a concentration of 1 mg. per 10 ml. of medium. Because of their low solubility in some cases the stock samples consisted of suspensions rather than solutions. The rates of growth found in the case of guanine, xanthine, caffeine, uric acid, allantoin, uracil, and guanosine were of the order of 0.1 mm. per hour, comparable to that obtained on the basal medium alone.

#### DISCUSSION

The results show that the deficient *Neurospora* mutant, strain 3254, is able to grow when adenine or the closely related purines, hypoxanthine or oxyadenine, are added to the basal medium. The rate of growth under these conditions is not comparable to that found for the original wild strain but is similar to that provided by a number of crude extracts including one from *Neurospora* itself which presumably contained all the constituents required for growth. Adenosine, adenosine-3-phosphate, and cozymase provided similar maximum rates of growth but were less efficient per unit weight than free adenine, the activity in each case being dependent on the amount of combined adenine known to be present. It appears, therefore, that this strain is a true adenine-deficient mutant and that the lower maximum rate of growth may be due to the growth characteristics of the strain itself.

The results obtained with oxyadenine are of interest because they show a close relationship between this purine and adenine. Although over 15 years have elapsed since oxyadenine was discovered in pig blood (10) and its occurrence as a nucleoside is known (11), little information has been contributed regarding its function or possible metabolism. As the function of adenine in the present mutant is no doubt concerned with the synthesis of nucleic acids and other adenine-containing compounds, it is likely that oxyadenine as well as hypoxanthine undergoes reduction to adenine in *Neurospora* metabolism.

While adenosine and adenosine-3-phosphate are readily diffusible and could be utilized directly for growth, the fact that they are only as active as the adenine present suggests that they may first be hydrolyzed to free purine. It is of interest that this mutant, unlike the pyrimidine-deficient mutants, can utilize ribonucleic acid directly.

The failure of guanine to provide for growth shows that this purine or its oxidation products, xanthine and uric acid, are not readily converted to adenine in this mutant. It is likely either that adenine is necessary for guanine synthesis or that the two compounds are synthesized by different mechanisms.

The lack of specificity shown by strain 3254 and the poor growth characteristics make it less desirable for assay purposes than the pyrimidine mutants previously described (6).

#### SUMMARY

Studies of the growth requirements of an experimentally produced *Neurospora* mutant, strain 3254, have shown that adenine, oxyadenine, and hypoxanthine are effective supplements to the basal medium of simple salts, sugar, and biotin. Adenine in combination as nucleoside, nucleotide, ribonucleic acid, or cozymase is also available for growth, but the relative activity of these compounds is dependent on the amount of adenine present. The rate of growth on extracts of several natural products including liver, malt, and *Neurospora* shows that one or more of the active compounds mentioned above is present in such preparations.

We should like to express our thanks to Dr. D. Klein for supplies of several liver fractions, to Dr. Mary V. Buell for the sample of oxyadenine, and to Dr. Paul D. Boyer for the sample of cozymase.

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# THE UTILIZATION OF ACETIC ACID FOR THE SYNTHESIS OF FATTY ACIDS\*

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The pertinent data on the synthetic mechanism employed by animals for the formation of fatty acids are both meager and indirect. Any hypothesis which deals with the mechanism of fat synthesis must take account of the fact that the constituent acids of the natural fats of mammals have an even number of carbon atoms and may include all homologues from  $C_4$  up to at least  $C_{20}$ .

The classic experiments of Knoop and Dakin with  $\omega$ -phenyl fatty acids suggested that oxidation of fatty acids proceeded by successive removal of  $C_2$  units, but no direct evidence was available as to the nature of the  $C_2$  unit. The isotope technique furnished direct proof that shortening of the carbon chain by 2 carbon atoms actually occurs (1) and that the fragment split off is acetic acid (2). It is well established that the normal animal can synthesize fatty acids from glucose. Various intermediates, notably pyruvic acid (3) and acetaldehyde (4), have been proposed.

In experiments in which acetic acid labeled by deuterium and heavy carbon was fed to mice and rats, we have obtained evidence (5) that fatty acids are synthesized by condensation of acetic acid, or of a compound into which acetic acid can readily be converted.

## EXPERIMENTAL

*Preparation of Isotopic Sodium Acetate*—Acetic acid containing 19.6 atom per cent excess  $C^{13}$  in the carboxyl group was prepared from methyl magnesium iodide and heavy  $CO_2$ , and converted to its sodium salt. Part of the sodium acetate was converted to sodium deuterio acetate by treatment with  $D_2O$  and active platinum at  $130^\circ$ . The methyl group contained 77 atom per cent excess deuterium.

*Feeding Experiments. Experiment A*—Two rats weighing 51 and 52 gm. were kept on a low fat, high carbohydrate diet. In addition, they received 1.6 mm of the isotopic acetate per 100 gm. of rat per day for 3 days. They gained an average of 11.5 gm. during the 3 day period. In this experiment the acetate was labeled with  $C^{13}$  only.

\* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation and from the Nutrition Foundation, Inc.

*Experiment B*—Five mice having an average weight of 21 gm. were kept on the same stock diet as the rats of Experiment A, with a daily addition of 1.6 mm of isotopic acetate per 100 gm. of weight for 8 days. This acetate was labeled with both  $C^{13}$  and D. 15 minutes before the mice were killed a sample of  $CO_2$  was obtained by passing the respiratory gas through a saturated solution of  $Ba(OH)_2$ . The  $BaCO_3$  contained 0.066 atom per cent excess  $C^{13}$ . The deuterium concentration of the body fluids was 0.09 atom per cent excess, determined by the usual procedure (6).

*Isolation of Tissue Constituents. Experiment A*—The fatty acids from the carcasses of the rats were isolated by the usual procedures. There were obtained 3 gm. of fatty acids which contained 0.040 atom per cent excess  $C^{13}$ . Part of the fatty acids was decarboxylated by heating with iron filing in a stream of nitrogen according to the procedure of Easterfield and Taylor (7). This carbon dioxide, derived from the carboxyl groups of the fatty acids, contained 0.090 atom per cent excess  $C^{13}$ . Glycogen, isolated from the pooled livers by the method of Ostern and Hubl (8), contained 0.020 atom per cent excess  $C^{13}$ . A sample of  $CO_2$  derived from the urea of the pooled urine by treatment with urease contained 0.033 atom per cent  $C^{13}$  excess. Since urea is formed from carbon dioxide (9), its isotope concentration should represent the average  $C^{13}$  concentration of the  $CO_2$  given off by these tissues during the experimental period.

*Experiment B*—The glycogen (13 mg.) obtained from the pooled livers of the mice contained 0.038 atom per cent excess  $C^{13}$ . From the pooled livers and pooled carcasses 225 mg. and 2.4 gm. respectively of fatty acids were isolated. The total fatty acids were analyzed for  $C^{13}$  and D. The saturated fatty acids of the liver fatty acids were obtained by way of the lead salts (10). Of the 48 mg. obtained, 4 mg. were employed for D and  $C^{13}$  analysis. The remaining 44 mg. were decarboxylated. The  $CO_2$  was converted to  $BaCO_3$  which in turn was transformed to  $CO_2$  for isotope analysis.

The fatty acids of the carcass were separated into saturated and unsaturated acid fractions. The saturated acids were analyzed for D and  $C^{13}$ . 22 mg. of  $BaCO_3$  were obtained by decarboxylation of 107 mg. of the saturated acids. The residue from the decarboxylation was treated with hydroxylamine to give the oximes of the ketones formed. The oximes contained 0.089 atom per cent excess  $C^{13}$ . The lead salts of the unsaturated fatty acids of the carcasses suffered considerable change during the 6 weeks they remained in the ice box before they were worked up, for, on partition between aqueous HCl and ether, about 40 per cent did not dissolve. 346 mg. of unsaturated fatty acids were obtained. They were dissolved in petroleum ether and brominated. After standing overnight a small amount of insoluble bromides was removed by filtration. The soluble bromides

were debrominated with zinc and the regenerated unsaturated acids oxidized in acetone solution according to the method of Armstrong and Hilditch (11). The monocarboxylic acid fraction which consists largely of pelargonic and heptonic acids was separated from the azelaic fraction by steam distillation. The monocarboxylic acid fraction was purified by two further steam distillations. On titration the mean molecular weight was found to be 148 as compared to molecular weights of 130 and 158 for heptonic and pelargonic acids respectively. This suggests that the mixture is composed of about 1 part of heptonic and 2 parts of pelargonic acid. The azelaic acid was isolated from the original oxidation mixture, purified by crystallization from water, and converted to its silver salt. Ag calculated, 53.6 per cent; found, 52.1 per cent.

The isotope concentrations of the various fatty acids and derived compounds are given in Table I. The  $C^{13}$  analyses were carried out on  $CO_2$  pre-

TABLE I  
*Isotope Concentrations in Lipids of Mice Fed Labeled Acetate*

The values are given in atom per cent excess.

	Carcass		Liver	
	$C^{13}$	D	$C^{13}$	D
Total fatty acids.....	0.081	0.13	0.103	0.32
Saturated fatty acids . . .	0.101	0.24	0.160	0.42
Carboxyl carbon of saturated fatty acids .	0.179		0.290	
Azelaic acid from "oleic" acid fraction .	0.090	0.14		
"Pelargonic" acid from "oleic" acid fraction	0.071	0.15		
Cholesterol	0.047	0.24	0.087	0.67

pared by the oxidation of 1 to 2 mg. samples of the fatty acids or by the decarboxylation of sufficient samples to yield from 2 to 4 mg. of  $CO_2$ . The deuterium analyses were carried out by a new micromethod requiring about 4 mg. of sample. This method will be described in another paper. The precision of this new method equals that of the falling drop method which we have previously employed. As the isotope concentrations in the lipids of the mice were higher than in the case of the rats, a more detailed study was made of the lipids of the mice.

#### DISCUSSION

The presence of both  $C^{13}$  and D in the total fatty acids after the feeding of labeled acetate demonstrates that both its carbon atoms are employed in the synthesis of some components of the total fatty acid mixture. As only small amounts of material were available, no pure compounds were isolated,



but the saturated fatty acids of Experiment B consist largely of palmitic and stearic acids. Similarly the azelaic acid and the monocarboxylic acid fraction obtained by the oxidative splitting of the "oleic" acid fraction must be derived principally from the oleic and palmitoleic acids of the unsaturated acid fraction. Since the deuterium concentrations of every sample analyzed are greater than that of the body fluids, there must have been a utilization of the methyl carbon of acetic acid for fatty acid formation. We are at a loss to explain the fact that in previous experiments in which deuterio acetate was fed the isotope concentrations of the carcass fatty acids were less than those of the body fluids. In those experiments the liver fatty acids were not investigated.

The presence of  $C^{13}$  in the fatty acids is proof that the carboxyl carbon of acetic acid is utilized for formation of the carbon chain of the fatty acids. We can eliminate the possibility that the incorporation of  $C^{13}$  resulted from the oxidation of acetate to  $CO_2$  and utilization of the labeled  $CO_2$  for the formation of a precursor of the fatty acids. The animals in Experiment B consumed about 13 gm. of diet and 1.6 mm of labeled sodium acetate per 100 gm. of weight per day. From the composition of the diet we calculate that it contained 6 gm. (500 mm) of carbon with an average  $C^{13}$  concentration of 0.064 atom per cent excess. In agreement with this calculated value we find that the  $C^{13}$  concentration of the respiratory  $CO_2$  at the end of the experimental period was 0.066 atom per cent excess. Since the isotope concentrations, not only in the carboxyl carbon atoms, but in the total fatty acids are higher, utilization of  $CO_2$  cannot explain these findings. The isotope concentration of both D and  $C^{13}$  is higher in the saturated fatty acids than in the total fatty acids, in agreement with results obtained in experiments in which heavy water was administered to mice (12).

The most significant feature of the data is the finding that the  $C^{13}$  concentration of the carboxyl carbon atoms of the saturated fatty acids is approximately twice as high as the average of all the carbon atoms in the saturated fatty acids. The most plausible distribution which will explain these data is one in which the labeled carbon is present at every other carbon atom; *i.e.*, at the odd numbered carbon atoms of the fatty acids. This hypothesis is supported by the finding that the "pelargonic" acid derived from carbon atoms 10 to 18 and the azelaic acid derived from carbon atoms 1 to 9 of "oleic" acids contain nearly the same  $C^{13}$  and deuterium concentrations.<sup>1</sup>

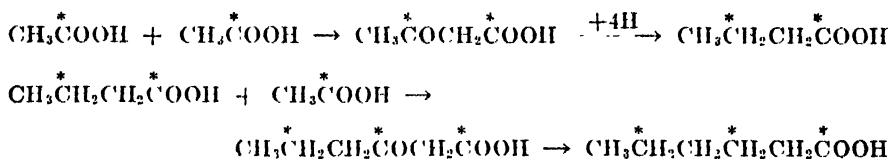
<sup>1</sup> On this hypothesis the  $C^{13}$  concentration of the pelargonic acid should be 80 per cent of that of the azelaic acid, since, of the 9 labeled carbon atoms of oleic acid, 5 should be located in the derived azelaic acid (carbon atoms 1, 3, 5, 7, and 9 of the oleic acid) and 4 in the derived pelargonic acid (carbon atoms 11, 13, 15, and 17 of the oleic acid). Taking into account the presence of palmitoleic acid has but a minor effect. The experimental value for the  $C^{13}$  concentration of the pelargonic acid is

The ketone remaining after decarboxylation of the carcass fatty acids of Experiment B contained 0.089 atom per cent excess  $C^{13}$ . From the isotope concentration of the original fatty acids (0.10) and of the carboxyl group (0.179) the isotope concentration of the ketone can be calculated on the assumption that the average chain length of the fatty acids was 17.

$$\text{Atom \% excess } C^{13} \text{ of ketones} = \frac{2 \times 17 \times 0.101 - 0.179}{33} = 0.099$$

Considering the fact that three isotope analyses are involved in this comparison, the 10 per cent discrepancy is not excessive.

The results clearly demonstrate that the labeled carbon is located in the chain as well as at the carboxyl carbon atom. It follows that the whole carbon skeleton of a fatty acid molecule can be formed from acetic acid, though the data do not demonstrate that the fatty acids are formed by this mechanism alone. The simplest explanation is that acetic acid condenses and is reduced to give fatty acids with an even number of carbon atoms. Our results do not give any information as to whether acetic acid itself or some compound into which it can readily be converted in the cell is the condensing unit. The biological condensation of acetate to yield acetoacetate has been established *in vivo* (13) as well as *in vitro* (14). Further condensation, either before or after reduction of the keto group, could reasonably yield the higher fatty acids.



The asterisks indicate the labeled carbon atoms.

While the carbon atoms of carbohydrates can serve as a source of the carbon chains of the fatty acids, there is no direct evidence as to the nature of the intermediates involved. It is generally assumed that pyruvate is one such intermediate. Findings from this laboratory indicate that in the liver only a small fraction of pyruvic acid is converted to acetic acid (15). No contradiction need exist with respect to the rôles of acetic acid and pyruvic acid in fat formation if it is assumed that pyruvic acid may be used directly for fatty acid synthesis without passing through the acetic acid stage. If pyruvate is used, a decarboxylation after the condensation to an acylpyruvic acid would be equivalent to the addition of acetate. The distribution of

79 per cent of that of the azelaic acid. Similar considerations require that the deuterium concentrations in the "pelargonic" and azelaic acids be the same. The data in Table I show this to be the case.

$C^{13}$  in the fatty acids requires that if indeed both pyruvate and acetate are employed for formation of the carbon chain of the fatty acids they must be used at random for the formation of every 2-carbon portion of the molecule. The occurrence of aldehydes of the higher fatty acids (16) and the possibility that they arise by reduction of the carboxyl group of the fatty acids or by decarboxylation of acylpyruvic acids suggest that aldehydes may be involved in the synthesis of fatty acids.

The isotope concentrations found in the cholesterol and the fatty acids are quite low, considering the high isotope concentration in the fed acetate. At least two factors are involved in this dilution: (a) acetate may not be the sole precursor of these products; (b) the dietary acetate is diluted by endogenous acetate.

Previous work in this laboratory has indicated that when 1.6 mm of acetate are fed to rats per 100 gm. of weight per day it is diluted 13-fold (15). While no data are available as to the corresponding dilutions in mice there can be little doubt that dilutions of similar magnitude occur in these animals. On this assumption the isotope concentrations of the acetate available to the cell are  $77/13 = 5.9$  atom per cent excess D and  $9.8/13 = 0.75$  atom per cent excess  $C^{13}$ . On the assumption that acetate is the only substance used for the synthesis of fatty acids, the maximum isotope concentration which would be found in the fatty acids after a very long time on a fat-free diet would be half of 5.9, or 3.0 atom per cent excess D, for  $CD_3COOH$  would be largely converted to  $-CD_2 \cdot CH_2-$ , since hydrogen used for reduction of the  $-COOH$  must be normal hydrogen arising from the body fluids. The corresponding  $C^{13}$  concentration of the fatty acids would be the same as that of the acetate, 0.75 per cent. During the 8 day period of Experiment B only half the fatty acids of the depots were replaced by newly synthesized fat (12), so that the isotope concentrations of the carcass fat could be only half of the maximum value cited above; namely, 1.5 atom per cent excess D and 0.375 atom per cent excess  $C^{13}$ . The saturated fatty acids of the carcass contained 0.24 atom per cent excess D and 0.101 atom per cent excess  $C^{13}$ . From the  $C^{13}$  data we thus estimate that at least one-fourth of all carbon atoms of the fatty acids are derived from acetate.

In confirmation of our previous results the cholesterol of the livers and carcasses of Experiment B were found to contain deuterium (17). The fact that the cholesterol also contained  $C^{13}$  is direct proof that in cholesterol synthesis the carbon, as well as the hydrogen, atoms of acetic acid are utilized. The isotope concentrations in this compound are higher than could be expected if they arose by introduction from the body fluids or the respiratory  $CO_2$ . This finding supports the thesis that conversions in which carbon atoms are utilized can be demonstrated by labeling with deuterium, provided that the isotope concentration in the conversion product exceeds

that of the body fluids. The stability of the C—D bond in such transformations depends upon the nature of the intermediate involved. For this reason a negative result may not be interpreted as disproving the existence of a postulated reaction.

Calculations similar to those above indicate that with an initial dilution factor of 13, and with consideration that in this 8 day period only 15 per cent of the body cholesterol is synthesized (12), it should contain  $3.0 \times 0.15 = 0.45$  atom per cent excess D and  $0.75 \times 0.15 = 0.113$  atom per cent excess  $C^{13}$ . As in the case of the calculations for the fatty acids, these values are predicated on the assumption that only acetate is used for cholesterol synthesis. The actual values found were 0.24 atom per cent excess D and 0.047 atom per cent excess  $C^{13}$ . The deuterium values indicate that at least half the cholesterol molecule is derived from acetate. If, as we have indicated in our previous papers, acetate is a compound which lies along the main route for the metabolism of some of the major components of the diet, we should expect more acetate to be produced per unit weight of mouse tissue than for that of rat tissue, for a mouse consumes more food per unit weight than a rat. These estimations suggest that acetic acid is an important source of the carbon atoms employed for the synthesis of fatty acids and a major source of the carbon atoms of cholesterol in the mouse. A similar conclusion had been reached with regard to cholesterol formation in rats (15).

The authors are indebted to Miss L. Ponticorvo and Mr. Irving Sucher for valuable assistance during the course of this work.

#### SUMMARY

1. Sodium acetate labeled with D and  $C^{13}$  was administered to mice and rats. The fatty acids and cholesterol were isolated separately from the livers and carcasses of the mice. Both lipids contained  $C^{13}$  and D, indicating the utilization of both carbon atoms of acetic acid in their formation. Degradation of the fatty acids suggests that the labeled atoms are distributed at alternate positions along the carbon chains.

2. The finding that the cholesterol contains not only deuterium but also  $C^{13}$  is proof that both carbon atoms of acetate are utilized in the synthesis of cholesterol.

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# ULTRAFILTRABILITY OF THIOURACIL IN HUMAN SERUM; DETERMINATION OF THIOURACIL

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This communication reports a study of the distribution of 2-thiouracil<sup>1</sup> between serum and serum ultrafiltrates at various hydrogen ion concentrations. Thiouracil was observed to be entirely ultrafiltrable when the serum was brought below pH 3. This fact permitted an analytical determination of thiouracil upon ultrafiltrates of acidified serum or plasma. Between pH 4 and 5.3 the fraction of thiouracil bound increased to about 60 per cent; from pH 6.3 to 7.0 the fraction bound appeared to be about constant, whereas a rapid decrease of ultrafiltrable thiouracil occurred between pH 7.0 and 7.8 (Fig. 1).

Electrometric titration of thiouracil (Fig. 2) was performed in an attempt to relate this behavior with proteins to changes in the state of charge of thiouracil. A single dissociating group with a  $pK'$  of 7.74 at 25° and an ionic strength of 0.16 was observed. The change in ultrafiltrability of thiouracil between pH 4 and 6.3 may perhaps be related to changes in the dissociation of groups upon the protein molecules, and the second rapid increase in bound thiouracil between pH 7.0 and 7.8 to the dissociation of thiouracil itself. However, the results obtained by varying the thiouracil concentration suggest that as the pH was increased in this latter range there was an increase in the concentration of groups upon the macro molecules which bound thiouracil. If the concentration of thiouracil was below certain critical levels (*e.g.*, about 3.7 mg. per cent at pH 7.55 at 25°, corresponding to pH 7.39 at 38°), 94 per cent or more of the thiouracil was bound (Fig. 3). Above these critical concentrations the type of group responsible for this binding appeared to be saturated, and a second type of group showing only a moderate affinity for thiouracil became evident. Hence the concentration of free thiouracil increased rapidly with increasing total thiouracil concentrations. Apparently the increase in binding of thiouracil between pH 7 and 7.8 represented mainly an increase in the concentration of the first type of group.

The observation that thiouracil binding is abolished by acidification suggested that the more acid deproteinizing agents might be the most useful in producing protein-free filtrates for thiouracil determination, contrary to the impression of Williams, Jandorf, and Kay (1). De-

<sup>1</sup> Supplied by the Lederle Laboratories, Inc., under the trade name, Deracil.

proteinization of serum or plasma by *p*-toluenesulfonic acid permitted quantitative recovery of added thiouracil (Table I). Recoveries after deproteinization by trichloroacetic acid were 65 to 100 per cent; by *p*-toluenesulfonic acid upon whole blood recoveries were 70 to 88 per cent. When *p*-toluenesulfonic acid is used, the results upon the serum of patients treated with thiouracil were in agreement with those obtained by ultrafiltration (Table II). The concentrations found were below 1 mg. per

TABLE I

*Recovery of Thiouracil Added to Plasma, Serum, Blood, and Serum Ultrafiltrates*

Thiouracil added to	Thiouracil added	Per cent recovery*		
		Direct determination	By ultrafiltration	After deproteinization by <i>p</i> -toluenesulfonic acid
	<i>mg. per cent</i>			
Ultrafiltrate.....	5.0	101		
“.....	3.0	98		
“.....	2.0	97		
“.....	1.0	96, 97		
Serum.....	3.0		98, 97, 103	
“.....	1.0		92, 100, 95	
“.....	0.5		94, 97, 97	
“.....	4.8			101, 98
“.....	4.5			103, 98, 98
“.....	4.0			103
“.....	3.5			92
“.....	3.0			101, 102
“.....	3.0†			99, 97†
“.....	2.5			101, 97
“.....	1.5			98, 98
“.....	1.0			95, 99, 104
Plasma.....	3.0			102, 100
Blood.....	5.0			88
“.....	3.5			73, 83

\* Each value represents a separate experiment.

† Samples kept 18 hours at room temperature over mercury at pH 7.50 and 7.72 before analysis, to simulate the conditions of ultrafiltration.

cent. At these low concentrations the precision of the ultrafiltration procedure was superior to that of the procedure employing protein precipitation, since dilution was avoided.

The color reaction of Grote (2) was applied, under conditions modified from those employed by Williams, Jandorf, and Kay (1), to the determination of thiouracil in *p*-toluenesulfonic acid filtrates and ultrafiltrates of serum. Grote's reagent has also been used by Danowski (3) and

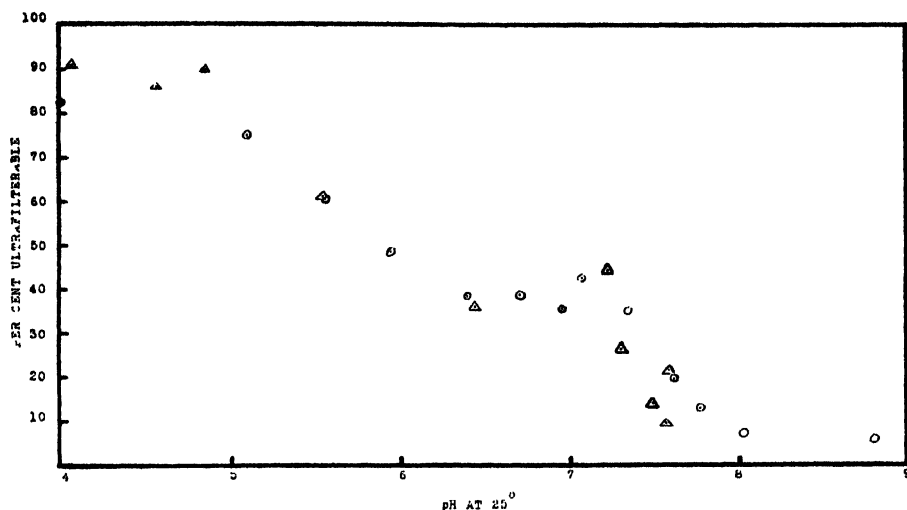


FIG. 1. Variation with pH of the per cent of serum thiouracil which was ultrafiltrable. Thiouracil concentrations, 5.3 to 5.45 mg. per 100 gm. of water.  $\circ$  serum from H. N. C.,  $\Delta$  serum from other normal donors. The Donnan effect upon the distribution of the anionic fraction of the thiouracil has not been considered.

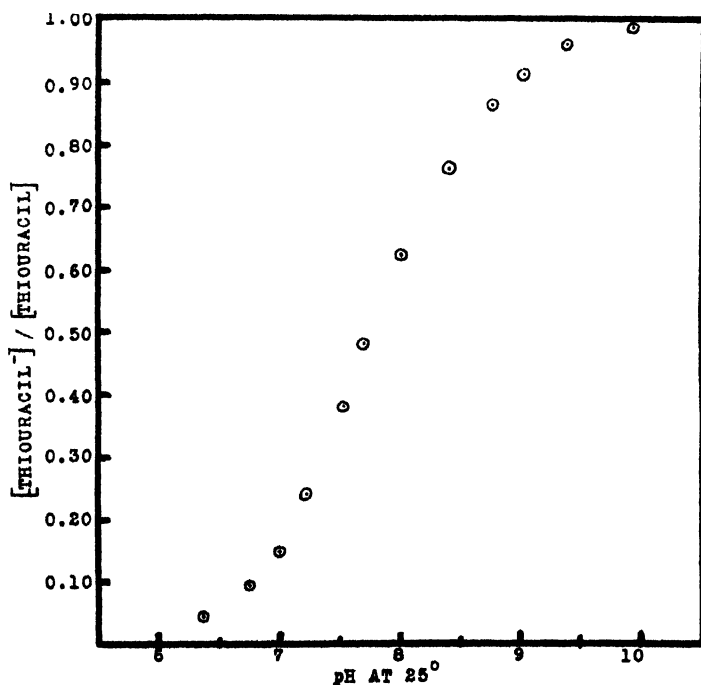


FIG. 2.  $\alpha$  curve for the titration of thiouracil at an ionic strength of 0.16



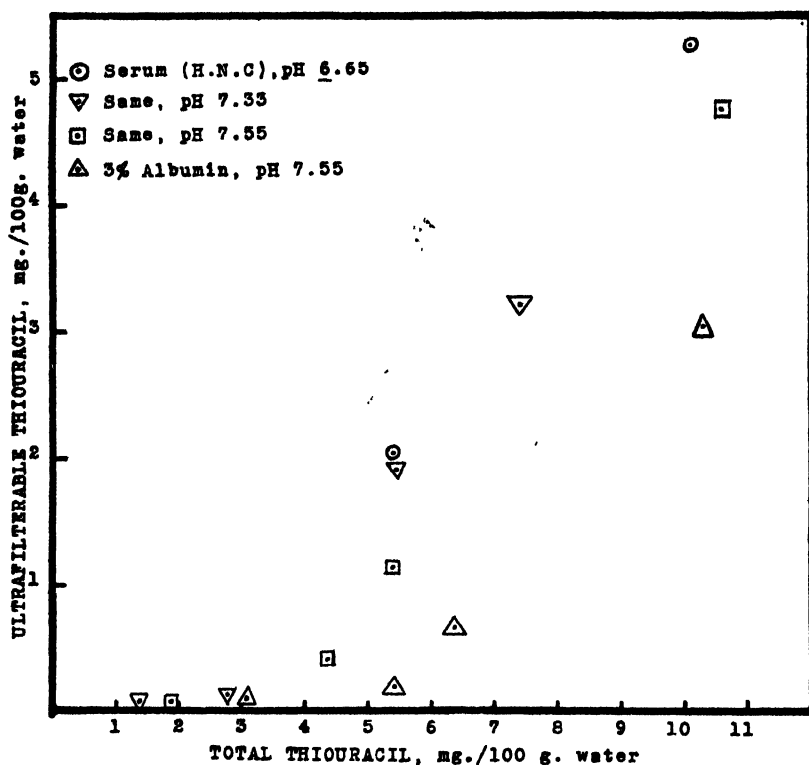


FIG. 3. Variation of ultrafiltrability of thiouracil with concentration

TABLE II

*Comparison of Results by Two Methods Applied to Subjects Treated with Thiouracil*

Subject No.	Dosage	Serum thiouracil	
		Deproteinization by <i>p</i> -toluenesulfonic acid	Ultrafiltration
		mg. per cent	mg. per cent
27984*	0.8 gm. per day	0.5	0.47
27984*	0.8 " " "	0.6	0.53
7030*	0.8 " " "	0.37	0.49
14092*	0.2 " " "	0.1	0.07
H. N. C.	0.4 " during previous 4 hrs.	0.60	0.56
"	0.05 gm. 2 hrs. previously†	0.23†	0.21†

\* Hyperthyroid patients.

† 6-Propylthiouracil, supplied through the kindness of Dr. E. B. Astwood.

Chesley (4) for the determination of thiourea. To avoid the necessity of precise pH adjustment and of electrometric pH measurements a barbitol

buffer was employed. Grote's reagent was modified by controlling the temperature during preparation, and by incubating it with glycine in the barbital buffer before use. Under the conditions employed this modified reagent has several advantages; most of the color of Grote's reagent was removed; the color with thiouracil developed more quickly and was deeper; the sensitivity of the color reaction to the presence of many substances was greatly reduced. The color formed by unmodified Grote's reagent with thiouracil in slightly buffered or unbuffered solutions adjusted to pH 8 to 9 was increased by the presence of small amounts of numerous agents (*e.g.*, glycine, glucose, ascorbic acid) and decreased by others (*e.g.*, barbital, ammonia, or borate buffers). The modified reagent was sufficiently insensitive to such interference to permit determinations upon serum ultrafiltrates. A pH of 8.0 was selected for the color development as giving a useful compromise between rate of color development and depth and stability of the color under the conditions employed.

#### EXPERIMENTAL

*Determination of Thiouracil; by Ultrafiltration through Cellophane*—To 3 ml. of serum or plasma was added 0.15 ml. of 3 N hydrochloric acid with shaking and the mixture introduced into an ultrafilter. The ultrafiltration technique illustrated by Danowski (3) has been used as well as the Laviertes ultrafilter (5). The former permitted a number of simultaneous filtrations; the latter was more convenient for occasional determinations. After filtering overnight, an aliquot (usually 2 ml.) of the ultrafiltrate was placed in a cuvette and sodium hydroxide added (about 0.03 milliequivalent per ml.) to bring the pH between 6 and 7, as indicated by a wide range indicator paper. Too high a pH resulted in turbidity. The color reagent was added as described below. The ultrafiltrate was diluted whenever the thiouracil level was above 1 or 2 mg. per cent.

*By Deproteinization by *p*-Toluenesulfonic Acid*—To 2 ml. of serum were added 6 ml. of water, and with good shaking, 2 ml. of 1 M *p*-toluenesulfonic acid. (This reagent solution was decolorized with norit in the course of its preparation.) After 20 minutes the solution was filtered through a 7 cm. Whatman No. 2 paper. By means of a graduated 0.1 ml. pipette 18 N sodium hydroxide was added to the filtrate until the pH fell between 5 and 7.5, spicules of a wide range indicator paper held in a forceps being used to check the acidity. Between 0.07 and 0.08 ml. of alkali was needed. With constant filtration technique the amount was nearly constant and the adjustment was usually made with three trials or less. Too high a pH produced turbidity. 5 ml. of the filtrate were measured into a cuvette. Two suitable reference standards were made with 0.1 to 0.5 ml. of a thioura-

cil standard (1 mg. per ml.) and a 0.2 M solution of sodium *p*-toluene-sulfonate to make 5 ml. 5 ml. of water were placed in another cuvette for a blank.

*Color Reagent; Grote's Reagent*—0.5 gm. each of sodium nitroprusside and hydroxylamine hydrochloride were dissolved in 10 ml. of water in a 50 ml. Erlenmeyer flask. 1 gm. of sodium bicarbonate was then added, and the flask rotated and placed in water at about 20° for 10 minutes. 0.1 ml. of bromine was added, and the flask shaken and kept at 37° for 20 minutes. Finally the solution was made to 25 ml. and stored at 5°.

*Buffer*—5.74 gm. of sodium barbital and 2.08 gm. of glycine were dissolved in about 450 ml. of water. 11.7 milliequivalents of hydrochloric acid were added to obtain a pH of 8.0 and the solution made to 500 ml. and stored at 5°.

The color reagent was made by mixing the two foregoing in the proportions of 1 part of the Grote's reagent and 9 parts of the buffer. The solution was aged about 1 hour at room temperature, or 25 minutes at 37° before use. The color passed from a purple-brown to a light yellow during this period. The rate of subsequent color development with thiouracil was increased, but the depth decreased by longer contact of the solutions. If the Grote's reagent was prepared in more dilute solution according to Chesley's procedure (4), little or no color was produced with thiouracil, indicating that the constituents producing color with thiourea and thiouracil are not identical.

*Color Development*—To the aqueous solutions in the cuvettes 40 per cent of their volumes of the color reagent was added. The transmittances were read at 660  $m\mu$  with a Coleman universal spectrophotometer, beginning 3 minutes after the addition and at 2 or 3 minute intervals until the absorptions were maximum. Ultrafiltrates developed full color in 5 to 10 minutes and then faded; other solutions developed maximum color in 8 to 20 minutes and showed slow fading. The rate of color development varied somewhat from batch to batch of reagent and with the room temperature. About seven cuvettes, including a blank, four unknown samples, and two standards, could be handled in a group. The plots of the log transmittance against concentration were nearly linear and passed close to the origin. Reliance could not be placed upon standardizations unless carried out simultaneously, although the variations in optical density were not large. Danowski (3) reached a similar conclusion using Grote's reagent for the determination of thiourea.

*Turbidity*—Correction for suspected turbidity in the solutions under colorimetric measurement was made as follows: After the minimum transmittance had been observed, a few crystals of chloramine-T were added to each tube to eliminate the green color, and the contents shaken well.

After 2 or 3 minutes the transmittances were observed again. Non-turbid solutions showed transmittances within 0.3 per cent of the blank.

Table I records recoveries of thiouracil added to serum ultrafiltrates and recoveries of thiouracil added to serum, plasma, and blood and determined by these two methods. Table II compares thiouracil analyses by the two methods applied to subjects treated with thiouracil.

*Electrometric titration of thiouracil* was carried out upon a 0.0039 M solution. Various quantities of 0.1 N acid and alkali were added to aliquots of this solution and the pH measured at 25° with a Cambridge Instrument Company laboratory model pH meter. Standard acetate buffer was taken to have a pH of 4.62. A titration carried out at an ionic strength of 0.16 is shown in Fig. 2. A single dissociating group with a pH of 7.74 at an ionic strength of 0.16 (7.78 in the absence of added sodium chloride) was observed.

#### *Diffusibility of Thiouracil*

Ultrafiltration was carried out anaerobically through cellophane with the Laviertes apparatus (5). Measured volumes of hydrochloric acid, water, and thiouracil solution were added to serum to produce a total dilution of 5 per cent. The pH was determined and the serum at once introduced into the ultrafilter. After 1 hour ultrafiltration was begun by raising one of the mercury bulbs. The filtrations were carried out at room temperature, which ranged from 20-25°. After 12 to 18 hours ultrafiltrates were analyzed by the procedure described above. In numerous cases the thiouracil and protein concentrations of the substrate were also measured to establish that thiouracil destruction was not measurable. Total serum proteins and albumin were determined by the method of Howe (6).

On 20 days, included in a 25 day period, serum was obtained from one donor for the above experiment. The results upon this serum are given in Fig. 1. Analysis at about the midpoint of the series gave a protein content of 6.7, and an albumin content of 4.0 per cent. Several other serum protein values of 6.7 to 6.9 per cent were obtained during this period. The variations in ultrafiltrability of thiouracil with pH observed on this serum at thiouracil concentrations of 5.30 to 5.45 mg. per 100 gm. of water describe a fairly smooth curve (Fig. 1). Results with the sera of a number of other normal persons are also given in Fig. 1, each point representing the serum of a different individual.

*Binding of Thiouracil by Serum Albumin*—Human serum albumin<sup>2</sup> was dialyzed for 48 hours against running distilled water, then made up to a

<sup>2</sup> Fraction V of human serum proteins, kindly supplied by Dr. Lawrence Oncley, Department of Physical Chemistry, Harvard Medical School.

concentration of 3 per cent, a pH of 7.55 at 25°, an ionic strength of 0.16 (with sodium chloride), and a known thiouracil concentration. After being left 1 hour in the Lavietes ultrafilter, a portion of the solution was ultrafiltered and the ultrafiltrate analyzed for thiouracil. Such albumin solutions showed a behavior toward thiouracil similar to that of serum (Fig. 3); however, 3 per cent albumin solutions showed a higher concentration of groups binding thiouracil with a high affinity than sera containing (after 5 per cent dilution) 6.5 per cent protein and 3.8 per cent albumin. One of the possible explanations for this disparity is that serum might contain substances competing with thiouracil for the binding groups. In this connection cholemic sera (Table III) were observed in several cases to bind less thiouracil than normal sera. Uremic sera (non-protein nitrogen 172 and 163 mg. per cent) resembled normal sera with regard to thiouracil binding.

TABLE III  
*Cholemic Serum and Thiouracil*

Patient No.	Diagnosis	Icterus index	Albumins	Globulins	pH of experiment, 25°	Thiouracil ultrafiltrable*	
						Found	Approximate average for normal subjects at this pH
			per cent	per cent		per cent	per cent
27760	Cholecystitis	120	1.7	7.0	7.33	50	31
27787	Acute infectious hepatitis	58	4.4	3.6	7.65	43	15
27787	" " "	28	4.2	3.0	7.63	24	16
28522	" " "	59	2.1	3.2	7.57	9.3	18
19830	Thyrotoxicosis; subacute hepatitis	123	1.8	4.6	7.56	32	19
19830	" "	110	2.3	3.5	7.21	47	35

\* Total thiouracil, 5.3 to 5.4 mg. per 100 gm. of water.

#### DISCUSSION

The extent to which thiouracil was ultrafiltrable appeared to undergo large changes in the range of pH encountered in human serum. Thus at a level of about 5 mg. per cent, the free thiouracil was nearly doubled by a fall of 0.2 pH unit and nearly halved by a rise of 0.2 pH unit. A mild acidosis or alkalosis might modify considerably the dynamics of thiouracil distribution and excretion. The relative increase produced by a pH decrease appeared to be particularly large in the range of 3 to 4 mg. per cent, and less at lower concentrations.

Williams, Kay, and Jandorf (7) and Williams (8) have reported the

whole blood levels of thiouracil reached with various dosages of thiouracil, and have observed that higher dosages produced only slightly higher blood levels than were produced by dosages of 0.2 to 0.4 gm. per day. Levels higher than 5 or 6 mg. per cent were infrequent. The foregoing data suggest an explanation for this finding: as the plasma thiouracil level increases and exceeds a critical level (which is determined by the concentration of groups binding thiouracil with a high affinity), there occurs a sudden increase in free thiouracil. Two effects limiting the concentration of circulating thiouracil may be expected: first, the uptake of thiouracil by tissue proteins will probably be increased; secondly, glomerular filtration of thiouracil will be increased with a possible increase in renal excretion.

The free plasma thiouracil probably represents the effective concentration of thiouracil in so far as the crossing of the capillary membranes and the cell walls (to the thyroid and other tissues) of the glomerular membranes and of the placenta is concerned. Whether the ability of this drug to combine with proteins represents a disadvantage or is the origin of its activity is not known.

#### SUMMARY

The distribution of thiouracil between serum and serum ultrafiltrates at varying concentration and pH has been studied. The diffusible portion ranged from about 100 per cent below pH 4 to about 5 per cent above pH 7.8. With increasing thiouracil concentration the ultrafiltrable fraction of thiouracil increased in a fashion suggesting that two types of groups with differing affinities were involved in binding thiouracil. Serum albumin solutions showed a similar behavior, although a higher concentration of binding groups was shown. Cholemic serum in several instances showed unusually high proportions of diffusible thiouracil.

An electrometric titration of thiouracil at 25° revealed a single dissociating group with a  $pK'$  of 7.74.

Two analytical procedures for the determination of serum thiouracil are described, one utilizing ultrafiltration of acidified serum, the other deproteinization by *p*-toluenesulfonic acid.

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# THE INABILITY OF HUMAN OR BEEF GLOBIN TO SUPPORT NORMAL HEMATOPOIESIS IN THE RAT WITHOUT ADDED ISOLEUCINE\*

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The fact that dried beef blood, added as the source of protein to an otherwise adequate synthetic diet, failed to support normal growth and hemoglobin regeneration in the rat has been demonstrated in earlier work in this laboratory (1, 2). The substitution of milk proteins for a part of the dried beef blood, on the other hand, permits good growth and the maintenance of normal hemoglobin formation, thus suggesting that the proteins of beef blood are of the "incomplete" type. Since whole blood contains a number of distinct proteins, it seemed necessary to investigate next the various isolated blood proteins for adequacy in supporting growth and hematopoiesis. The protein, globin, was selected first, since it is quantitatively the predominant protein of whole blood.

Preliminary results (3) indicated that neither purified human nor beef globin was adequate for supporting a normal rate of growth or hemoglobin formation in the rat. After the globin-containing diet had been fed for 4 to 6 weeks, a rapid decrease in body weight occurred, an anemia developed, and death invariably resulted.

While this investigation was in progress, a report of the amino acid composition of beef hemoglobin appeared (4), showing that this protein contains a relatively small amount of isoleucine. Some time later two separate investigations (5, 6) demonstrated that hemoglobin or globin, fed as the source of protein in an adequate diet, failed to support growth in the rat, whereas the addition of isoleucine promptly resulted in a satisfactory increase in body weight. Preliminary results in this laboratory (3) confirmed this observation.

The present investigation was designed to determine the effect of isoleucine on hematopoiesis in anemic rats fed a synthetic diet containing purified human or beef globin as the protein.

## EXPERIMENTAL

21 day-old male rats, weighing from 40 to 50 gm., of the Connecticut Agricultural Experiment Station strain were used. They were housed in

\* Aided by a grant from the Griffith Laboratories, Chicago, Illinois.



individual cages and fed a synthetic diet having the following per cent composition: purified human<sup>1</sup> or beef globin 19.6,<sup>2</sup> sucrose 10, dextrin 39.4, hydrogenated cottonseed oil 27, salt mixture (Wesson (7)) 4. The globin preparations were made by the method on Anson and Mirsky (8). Control rats were fed the same synthetic diet, except that casein (22.5 per cent) was used as the protein. All animals received the following vitamin supplements: daily, 200 mg. of ryzamin-B<sup>3</sup> and 200 mg. of liver extract;<sup>4</sup> twice weekly, 3 drops of fortified haliver oil.

Body weights were recorded weekly and hemoglobin determinations were made biweekly on blood taken from a tail vein. A photoelectric acid-hematin method, which had been carefully checked with samples of known hemoglobin content (O<sub>2</sub> capacity method), was used for obtaining hemoglobin values.

After the rats had been fed the globin diet for 4 weeks, supplementation with isoleucine<sup>5</sup> was started. This interval was arbitrarily chosen, since it permitted sufficient time for the development of a distinct anemia but was not so long as to result in the complete loss by death of the animals. Isoleucine (*dl*-) was fed at a level of 100 mg. daily, an amount approximately double that consumed in casein by the control rats. The doubled amount of isoleucine was used to allow for the presence of the non-utilized *d* isomer (9). The isoleucine was incorporated directly in the globin-containing synthetic diet. The amount of the diet allowed each rat daily was restricted to the average daily amount, found to be 1.4 gm., ingested prior to supplementation. The purpose of the food restriction was to prevent a probable increase in food consumption following supplementation, a circumstance which would have introduced undesired variables in the experiment.

Supplementation with isoleucine was continued for a 6 week period and then discontinued. Weekly body weight and hemoglobin determinations were made during the period of supplementation and then following isoleucine withdrawal until the experiment was terminated by the death of the animals.

<sup>1</sup> Kindly supplied by Dr. N. S. Ferry and Dr. H. B. Devlin, Parke, Davis and Company, Detroit.

<sup>2</sup> This amount furnished 18 per cent protein, allowing an average of 8.0 per cent moisture and ash found in the globin preparations employed.

<sup>3</sup> Burroughs, Wellcome and Company, Inc., Tuckahoe, New York. Appreciation is expressed to Mr. C. M. Cypher for supplying a portion of the material used.

<sup>4</sup> Wilson's liver fraction B. Appreciation is expressed to Dr. David B. Klein for a generous supply of this material.

<sup>5</sup> *dl*-Isoleucine, Merck and Company. Appreciation is expressed to Dr. D. F. Robertson for supplying this substance.

*Results*

The average body weights, at biweekly intervals, of the control and globin-fed groups of rats are given in Table I. It is evident that the control rats grew at the rapid rate characteristic of the strain used, whereas the animals fed either human or beef globin lost weight. Death occurred in all of these rats, not given isoleucine supplementation, within 6 weeks.

The group average hemoglobin values, with minimum and maximum values obtained on individual rats, are given in Table II. The normal

TABLE I  
*Average Body Weights of Control Rats and of Rats Fed Human or Beef Globin*

Protein fed (18 per cent level)	Initial No. of rats	Body weight			
		0 wk.	2 wks.	4 wks.	6 wks.
		gm.	gm.	gm.	gm.
Casein.....	12	42	124	193	297
Human globin . . . . .	40	43	35	32	28
Beef " . . . . .	10	44	37	33	

TABLE II  
*Hemoglobin Content of Blood of Control Rats and of Rats Fed Human or Beef Globin*

Protein fed	Initial No. of rats	Hemoglobin*			
		0 wk.	2 wks.	4 wks.	6 wks.
		gm. per cent	gm. per cent	gm. per cent	gm. per cent
Casein . . .	12	10.8 (9.2-13.3)	13.6 (9.2-14.3)	13.7 (11.4-14.9)	14.8 (13.3-15.8)
Human globin . .	40	11.3 (9.1-14.6)	13.4 (5.7-16.8)	9.3 (3.0-13.6)	3.2
Beef globin.	10	12.1 (8.8-14.2)	13.1 (9.4-16.0)	9.4 (2.4-12.3)	

\* Group averages, with minimum and maximum values on individual rats.

increase in hemoglobin with age (10) was found in the control rats, while a distinct anemia developed in both groups of globin-fed animals. The anemia usually became severe before the death of the animal.

Hemoglobin values for the control rats and for the globin-fed rats given isoleucine are given in Table III. The values for the control rats increased somewhat as the experiment progressed until a normal mature level was attained (10). Isoleucine supplementation was followed in every animal, fed either human or beef globin, by a steady rise in hemo-

TABLE III  
*Hemoglobin Content of Blood of Control Rats and of Rats Fed Human or Beef Globin with and without Isoleucine Supplementation*  
 The values represent group averages for hemoglobin, with minimum and maximum values on individual rats, given in gm. per cent.

Protein fed	4 wks.	5 wks.	6 wks.	8 wks.	9 wks.	10 wks.	11 wks.	12 wks.	13 wks.	14 wks.	15 wks.	16 wks.	17 wks.	18 wks.
Casein (12 rats)	13.7 (11.4-14.9)		14.8	15.0		15.3 (14.1-16.9)		15.2		15.5		16.1		16.0 (14.8-18.0)
	Isoleucine added							Isoleucine withdrawn						
Human globin (12 rats)	9.3 (8.9-13.6)	12.3	14.5	16.0	15.9	15.6 (14.6-17.1)	14.1	12.1	11.2 (9.7-12.9)	11.3	10.1	9.2	8.3	10.4*
Beef globin (7 rats)	9.4 (8.0-13.3)	11.5	14.0	14.8	14.8	15.4 (15.0-15.8)	14.2	12.8	12.2 (11.7-13.4)	8.1*				

\*Data obtained on one surviving rat.

globin concentration and a normal adult value was attained within 4 weeks. This level was maintained during the remainder of the 6 week period.

The withdrawal of isoleucine was followed by a gradual, consistent decrease in hemoglobin values. Mild to severe anemia developed in the animals, severe anemia usually preceding the death of the animal, as before. Death of all rats occurred within 3 to 8 weeks after isoleucine supplementation was discontinued.

The body weights (data not included) of the globin-fed rats showed a slight gradual increase following isoleucine supplementation. As would be expected, the increase was small (the average weight increase was 8 gm. in 6 weeks) because of the restriction of food intake. Following isoleucine withdrawal there was a gradual decrease in body weight until the death of the animal occurred.

#### DISCUSSION

The present observations offer evidence that neither human nor beef globin fed as the sole source of protein in an otherwise adequate diet will support normal hematopoiesis in the rat, but that supplementation with isoleucine is required. This finding adds another example of an "incomplete protein" failing to promote normal hemoglobin formation. The feeding of deaminized casein produces an anemia in the rat unless lysine is added (11). A quantitative lack of protein is also known to produce a mild chronic anemia in the rat (12, 13).

The present work also adds isoleucine to the list of amino acids now known to be essential for hemoglobin formation, the others being lysine (11) and tryptophane (14).

The finding that isoleucine is needed for hemoglobin formation in the rat raises an interesting question. Either the globin of rat hemoglobin, unlike that of human or beef hemoglobin, contains isoleucine or the present data demonstrate the need of an amino acid (isoleucine) for the synthesis of a protein deficient in the amino acid in question. Such a circumstance would suggest that hemoglobin may be synthesized in the rat by way of an as yet unidentified intermediate which contains isoleucine. This possibility merits further careful investigation.

The results of the present investigation may seem at variance with the observations of Whipple and his associates (15) that hemoglobin (or globin) is well utilized for the formation of new hemoglobin in the hemorrhagic anemia of the dog. However, it must be remembered that a different experimental procedure and a different species of animal was employed in the study in question than in the present one. Also, it is possible that isoleucine may have been derived from the dog's own tissue

protein, since a loss of weight occurred in the dogs in the investigation under discussion.

Another point upon which the results of the present study may have a bearing is in the use of globin preparations as a blood substitute (16). The present data indicate that globin, while it may have temporary value as a blood substitute, probably could not be relied upon over any protracted period to furnish the amino acids needed for hemoglobin or other body protein formation unless it is supplemented with isoleucine.

#### SUMMARY

Purified human or beef globin fails to support growth and normal hemoglobin formation in the rat.

Supplementation of either type of globin with isoleucine, all other known dietary factors remaining unaltered, results in the maintenance of a normal concentration of hemoglobin in the blood.

The subsequent removal of isoleucine is followed by the development of a mild to severe anemia and death of the animal.

These observations indicate that isoleucine is needed for normal hematoipoiesis in the rat.

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# THE UTILIZATION OF *d*-AMINO ACIDS BY MAN\*

## V. HISTIDINE

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The recent findings (1, 2) that *l*(-)-histidine is not required for the maintenance of nitrogen balance in man prompted us to investigate the metabolic fate of *d*(+)-histidine in the human. The excretion procedure which we have previously employed (3) for determining the degree of utilization of *d*-amino acids seemed uniquely suited in this instance in which the nitrogen balance technique would not be effective. Five feeding experiments showed that within 9 hours of the administration of 0.01 mole of *dl*-histidine·HCl·H<sub>2</sub>O (equivalent to 775 mg. of *d*(+)-histidine)  $692 \pm 35$  mg. more histidine were excreted in the urine than after the feeding of 0.01 mole of *l*(-)-histidine·HCl·H<sub>2</sub>O. This difference in histidine output which is equivalent to  $89.2 \pm 4.5$  per cent of the *d* form ingested and the finding that on isolation this excess histidine proved to be of the *d* configuration suggest that *d*(+)-histidine is poorly or not at all utilized by man.

The analytic procedure for the colorimetric estimation of histidine in the urine which was developed for this work is described in detail.

## EXPERIMENTAL

*Preparation of dl-Histidine Monohydrochloride Monohydrate*—The racemate was prepared by a modification of the procedure of Bergmann and Zervas (4) from *l*(-)-histidine·HCl·H<sub>2</sub>O, Merck, containing 20.02 per cent N by micro-Kjeldahl analysis (5) and having a specific rotation<sup>1</sup> of  $[\alpha]_D^{20}$  (1.52 gm. in 100 cc. of 1 N HCl) =  $+8.10^\circ$ . A mixture of 21.0 gm. (approximately 0.1 mole) of *l*(-)-histidine·HCl·H<sub>2</sub>O, Merck, 50 cc. of glacial acetic acid, and 13.5 cc. of acetic anhydride was heated on the steam bath for 2 hours. The excess of acetic acid was then removed by

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<sup>1</sup> In aqueous solution *l*-histidine is levorotatory and *d*-histidine is dextrorotatory. When these compounds are dissolved in HCl, the respective rotations are reversed. To avoid confusion naturally occurring histidine will be referred to as the *l*(-) isomer and the unnatural as the *d*(+) isomer.

concentration of the reaction mixture *in vacuo* to a thick syrup three times successively, after the addition of water. The final product was dissolved in 100 cc. of 6 N HCl and hydrolyzed by boiling under a reflux in an all-glass apparatus for 20 hours. The excess of hydrochloric acid of this solution was removed by three evaporations *in vacuo* following 50 cc. additions of water. The final syrup was dissolved in 50 cc. of 95 per cent ethanol and the monohydrochloride was precipitated from solution by the gradual addition of a 1:1 mixture of pyridine and ethanol. After 72 hours of refrigeration the crude crystalline product was collected on a small Büchner funnel and was purified by two recrystallizations from water and alcohol (1:2 volumes). The refined substance weighed 12.0 gm., contained 20.03 per cent N, and was optically inactive in aqueous or 1 N HCl solution.

*Colorimetric Estimation of Histidine in Urine*—A careful experimental survey of the available methods for the determination of urinary histidine led us to the development of a procedure based on the intensity of the red color produced in the urine sample by the Pauly diazo test (6) following the destruction of the interfering phenols by the Kapeller-Adler permanganate reaction (7). Recovery tests with urine specimens and protein hydrolysates have shown that the technique is suitable for accurate analytical work.

*Reagents*<sup>2</sup>—

*Sulfanilic acid reagent.* 2.5 gm. of sulfanilic acid were dissolved in 25 cc. of concentrated hydrochloric acid and 200 cc. of water by heating and stirring. The solution was then cooled and the volume adjusted to 500 cc.

*Sodium carbonate, 20 per cent solution.*

*Sodium nitrite, 2 per cent solution.*

*Potassium permanganate reagent.* 1.58 gm. of the salt were dissolved in 100 cc. of 0.1 N sulfuric acid.

*Sulfuric acid, 0.2 N solution.*

*Histidine standard stock.* 13.5 mg. of *l*(-)-histidine·HCl·H<sub>2</sub>O (20.02 per cent N found) were dissolved in 200 cc. of water. 1 cc. of this solution is equivalent to 0.05 mg. of histidine.

*Procedure*

To 1 cc. of urine sample usually prepared by a 1:5 dilution of normal human urine in a 10 cc. graduated Klett-Summerson photoelectric colorimeter tube is added 1 cc. of 0.2 N sulfuric acid. Acid permanganate reagent is added dropwise to this sample with shaking until the deep rose persists. This is followed by the addition in succession of 1 cc. of sodium

<sup>2</sup> Merek reagents were used throughout.

nitrite solution and 1 cc. of sulfanilic acid reagent. After 5 minutes, 1 cc. of sodium carbonate is added to the reaction mixture which 2 minutes later is made to the 10 cc. mark with water. The resulting color is read within 15 minutes in the Klett-Summerson photoelectric colorimeter with Filter S-54 and compared with that of 1 cc. of histidine standard similarly treated. The amount of histidine in the sample can also be estimated from a previously prepared calibration curve.

### Results

The suitability of the procedure was determined by submitting varying amounts of histidine standard to the diazo reaction before and after treat-

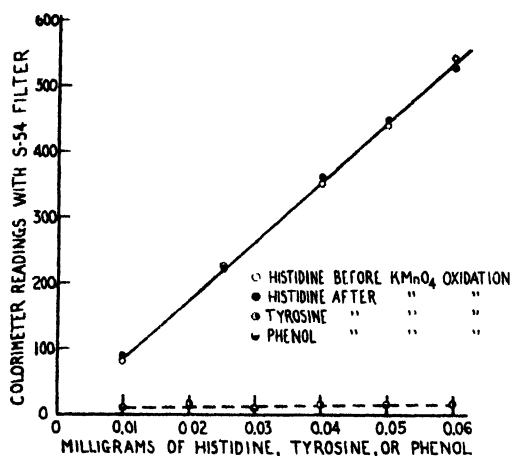


FIG. 1 Relation of color intensity to amount of histidine, tyrosine, or phenol. Each point represents the average value of ten determinations. The lines were drawn from computation of the data by the method of least squares.

ment with acid permanganate reagent. The results of these experiments together with those on phenol and tyrosine solutions after the permanganate reaction are shown in Fig. 1. It is evident from the coincidence of the histidine tests that histidine itself is not oxidized by this reagent, whereas the interference from phenol and tyrosine is nullified by this reaction. Also the linear relationship of the color intensity to the amount of histidine is interpreted as evidence of the validity of Beer's law for the color reaction.

The applicability of the test to the estimations of histidine in human urine and protein digests is demonstrated by the recovery test data shown in Table I. Quite apart from the immediate object of this paper, it is of interest to note that, although the histidine N of total N values found by



this technique are somewhat higher than the 5.36 per cent for casein and 1.14 for gelatin previously found by one of us employing the electrolytic method (8), they fall in the range reported by others (9). Analyses of 24 hour urine specimens of 50 normal adult males (60 to 110 kilos) on normal diets by this procedure have shown the daily histidine output to vary from 180 to 760 mg. This represents an excretion of 0.09 to 0.58 mg. of histidine per cc. of urine, which falls within the range 0.09 to 1.02 mg. of histidine per cc. of urine found by Langley (10) and the range 0.06 to 0.76 mg. of histidine per cc. of urine reported by Racker (11). It is also to be noted that the individual human histidine output shows marked diurnal variation and that histidine is excreted by the human in much larger amounts than are the other amino acids (12).

TABLE I  
*Recovery of Histidine Added to Urine and Protein Hydrolysates*

Sample	Histidine added mg.	Histidine found mg.	Recovery of added histidine per cent	Histidine N of total N per cent
Urine A, 0.2 cc. ....	0	0.0154		
" " 0.2 " ....	0.05	0.0655	100.1	
" B, 0.2 " ....	0	0.0468		
" " 0.2 " ....	0.025	0.0720	100.3	
Amigen, 0.102 mg. N per cc. . . . .	0	0.0198		5.3
Urine C, 0.2 cc. ....	0	0.0165		
" " 0.2 " + amigen, 0.102 mg. N. . . . .	0.0198	0.0362	99.5	
Casein, Harris, HCl digest, 45.4 mg. N per cc.*.	0	0.0391		5.8
" " " " 45.4 " " " "	0.05	0.0891	100.0	
Gelatin, U. S. P., HCl digest, 73.4 mg. N per cc.*.	0	0.0168		1.6
" " " " 73.4 " " " "	0.05	0.0665	99.4	

\* 1 cc. of 1:250 dilution used for this analysis.

### *Human Experiments*

Three subjects were given by mouth 0.01 mole (2.1 gm.) of *l*(-)- or *dl*-histidine·HCl·H<sub>2</sub>O dissolved in 240 cc. of water following the elimination of the zero hour specimen which was collected 2 hours after a light breakfast. 120 cc. of water were also given at the end of each of the succeeding 9 hours of the experimental period. The urines were then collected hourly after zero time and all collections analyzed for histidine. The control experiments were similarly performed except that no histidine was fed. Moreover, since the ingestion of food did not seem to affect appreciably the output of histidine, the fast was maintained for the first 3 hours of the experiments only.

The effect of the administration of the histidine isomers on the histidine

level in the urine is shown in Fig. 2. A tabulation of the data from four other experiments is shown in Table II. It will be noted from these data that the ingestion of 2.1 gm. of *l*(-)-histidine produces only a slight increase over the normal histidine output, whereas the feeding of the racemate results in a 10-fold or greater increment of the normal histidine output. In order to ascertain the approximate amount of urinary histidine arising from the ingestion of the *d*(+) variety, it seemed reasonable to subtract the total histidine output for the 9 hour period following the administration

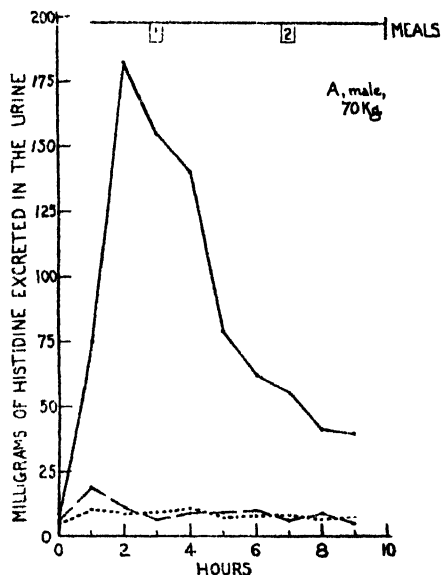


FIG. 2. Urinary output of histidine of Subject A, after administration of 0.01 mole (2.1 gm.) of *l*(+)- and *dl*-histidine·HCl·H<sub>2</sub>O. The solid line indicates excretion after ingestion of the *dl* form, the broken line after ingestion of the *l* isomer, and the dotted line normal output. Meals 1 and 2 had approximately the same composition in all experiments.

of the naturally occurring variety from the histidine output of the 9 hour interval following the ingestion of the racemate. The values so obtained are listed under the heading (b) - (a) in Table II. Calculations made from these data reveal that 81.5 to 97.2 per cent of the 775 mg. of *d*(+)-histidine which are contained in the 2.1 gm. of the ingested *dl*-histidine·HCl·H<sub>2</sub>O is excreted within 9 hours after administration. This indicates that *d*(+)-histidine is poorly or not utilized at all by man.

**Isolation of *d*(+)-Histidine from Human Urine after Administration of *dl*-Histidine**—To 1280 cc. of pooled urine which was collected in the 6 hour period after administration of 0.01 mole of *dl*-histidine·HCl·H<sub>2</sub>O to two subjects and found to contain 913 mg. of histidine by colorimetric analysis

were added with vigorous stirring 64 gm. of finely powdered mercuric chloride (5 gm. per 100 cc.). On adjusting the solution reaction to pH 5 to 6 with 20 per cent sodium carbonate a heavy white precipitate formed which was filtered off by suction after 1 hour. This precipitate was dissolved in 150 cc. of 1 *N* HCl and the mercury salt was decomposed by saturation of the solution with H<sub>2</sub>S. After clarification with norit A, analyses showed that the filtrate from this procedure contained 865 mg. of histidine or 95 per cent of that originally present. The excess of hydrochloric acid of this solution was removed by three evaporations *in vacuo* following additions of water. The final syrup was then dissolved in 25 cc. of 95 per cent ethanol and the histidine monohydrochloride was precipitated from solution by the slow addition of a 1:1 mixture of pyridine and ethanol. After 24 hours of refrigeration the crude crystalline product was collected

TABLE II

*Total Urinary Histidine for 9 Hour Period following Administration of 0.1 Mole of l(-)- or dl-Histidine*

2.1 gm. of the hydrated histidine chloride (equivalent to 1.55 gm. of histidine) were fed in each experiment.

Subject, sex, and body weight	Normal histidine output	Histidine output after administration of isomers		(b) - (a)	Recovery in urine of d form fed
		l(-) (a)	dl (b)		
	mg.	mg.	mg.	mg.	per cent
A, male, 70 kilos . . . . .	87	93	846	753	97.2
" " 70 " . . . . .	75	103	804	701	91.0
F, female, 60 kilos . . . . .	51	96	727	631	81.5
I, " 65 " . . . . .	63	119	801	682	88.0

on a micro Büchner filter and was purified by two successive recrystallizations effected by dissolving the product in 10 cc. of hot water and precipitation from the hot solution by the gradual addition of 20 cc. of 95 per cent ethanol. The refined product weighed 604 mg. which on characterization by the histidine content (446 mg.) as determined by our colorimetric procedure, the nitrogen content, and the optical activity in 1 *N* HCl solution gave evidence of its identity with d(+)-histidine·HCl·H<sub>2</sub>O.

C<sub>6</sub>H<sub>9</sub>N<sub>3</sub>O<sub>2</sub>·HCl·H<sub>2</sub>O. Calculated, C<sub>6</sub>H<sub>9</sub>N<sub>3</sub>O<sub>2</sub> 73.9; found, 73.7

" N 20.04; found, 20.00

Cox and Berg (13), [α]<sub>D</sub><sup>25</sup> = -8.25°; found, -8.35°

These results clearly point to the conclusion that the excess of urinary histidine resulting from the administration of the racemate is of the *d* variety.

Comment

Although *d* and *l* forms of histidine have been reported to be equally effective in promoting growth in mice (14) and rats (13), data obtained from excretion experiments suggest that the *d* variety is poorly utilized by guinea pigs (15), rabbits (16), and dogs (17). The present studies indicate that the *d* component of orally administered *dl*-histidine is poorly metabolized or not metabolized at all by man. This finding seems to us remarkable in view of the observations that *l*(-)-histidine is not a dietary essential for the maintenance of the N balance in adult man. In this connection, we also wish to note that none of the urines collected after administration of *l*(-)- or *dl*-histidine gave the atypical green color with the indican test which we reported to occur with the urines of adult human males on a histidine-deficient diet (2).

SUMMARY

It has been found that within 9 hours after administration of *dl*-histidine to adult humans a total excess of urinary histidine is excreted which is approximately equal to the amount of *d* component so ingested. This observation and the optical activity of an isolated fraction of this excess histidine suggest that *d*(+)-histidine is poorly utilized by man.

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# THE DETERMINATION OF OXYBIOTIN IN THE PRESENCE OF BIOTIN\*

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The biological activity of oxybiotin (1, 2) and of an apparently similar substance (3) has been recently described. Prior to these reports the only compound possessing yeast growth activity comparable to that of biotin was desthiobiotin (4), which exerts its biological activity by virtue of the fact that it is converted into biotin by the yeast cell (5, 6).

It was of importance to determine whether oxybiotin is likewise transformed into biotin by microorganisms or whether it has intrinsic activity. In order to investigate this point, an assay procedure was needed for the determination of oxybiotin in the presence of biotin.

Since all of the organisms tested so far are stimulated by oxybiotin, it appeared unlikely that an organism could be found readily which would not respond to the oxygen analogue and thus provide the basis for a microbiological differential assay. A chemical method was therefore developed which is based on the different behavior of ethers and thio ethers toward oxidizing agents.

It has been shown previously that potassium permanganate oxidizes biotin to the corresponding sulfone (7). This latter substance has a low degree of biotin activity in the presence of aspartic acid, and is inactive when this amino acid is not included in the medium (8). Oxybiotin could be expected to be stable toward dilute permanganate. It has now been observed that dilute potassium permanganate inactivates biotin completely in pure solution, as well as in extracts or hydrolysates of biological materials, while oxybiotin is virtually unaffected by such treatment. Microbiological assay of permanganate-treated materials makes it possible to determine their oxybiotin content. By the use of an assay procedure, based on the foregoing considerations, it has been demonstrated that *Saccharomyces cerevisiae* and *Rhizobium trifolii* utilize the oxybiotin molecule as such, and do not transform it into biotin.

## EXPERIMENTAL

*Action of Potassium Permanganate upon Pure Solutions of Biotin and Oxybiotin*—In developing the present assay method, a study was made of

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the stability of biotin and oxybiotin toward dilute potassium permanganate solutions. 10 ml. of a solution containing 1.0 millimicrogram of *d*-biotin, or 4.0 millimicrograms of *dl*-oxybiotin, were treated for 5 to 10 minutes at room temperature with 10 ml. of a 0.01 *N* solution of potassium permanganate. The excess of the permanganate was decolorized by the careful addition of 0.1 *M* sodium sulfite, and the solution diluted to 100 ml. with distilled water and assayed by the Hertz (9) modification of the yeast growth method of Snell, Eakin, and Williams (10).

As may be seen in Fig. 1, the biotin activity is completely destroyed by permanganate, while oxybiotin suffers only a slight inactivation by this treatment.

*Recovery of Biotin and Oxybiotin Added to Commercial Yeast Autolysate* - In order to extend the above result to biological materials, experiments

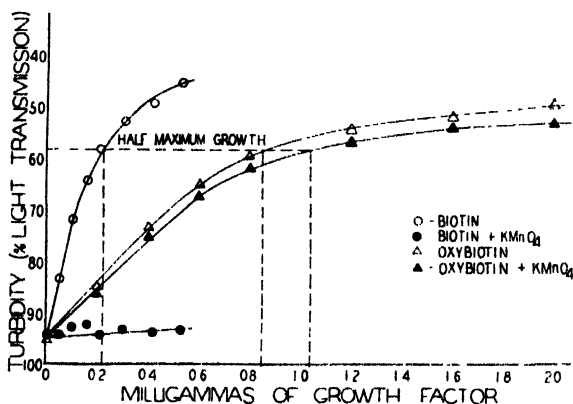


FIG. 1. Yeast growth curves obtained with untreated and with permanganate treated biotin and oxybiotin solutions.

were conducted in which known amounts of *d*-biotin and *dl*-oxybiotin were added to a commercial yeast preparation (Difco autolyzed yeast), and the amounts of added growth promoter determined.

A known amount of *dl*-oxybiotin was added to a suspension of 0.5 gm. of Difco autolyzed yeast in 10 ml. of distilled water. 5 ml. of 9 *N* sulfuric acid were then added, and the mixture was autoclaved for 90 minutes at 15 pounds pressure. The resulting hydrolysate was washed into a graduated 50 ml. centrifuge tube, and made up to 50 ml. with distilled water. The cell debris was sedimented by a short centrifugation, and a 5 ml. aliquot of the clear supernatant was withdrawn and diluted to either 50 or 100 ml. with distilled water, depending upon the concentration of growth factor. 10 ml. of this solution were treated with 0.01 *N* permanganate, followed by 0.1 *M* sodium sulfite as described in the preceding section. The pH was

adjusted to approximately pH 4.0 by the addition of 0.5 N potassium hydroxide. The solution was made up to 100 ml. with distilled water, and assayed microbiologically (9). Throughout this paper, the amounts of oxybiotin are expressed in terms of the *dl* form, based upon a standard *dl*-oxybiotin growth curve.

Table I shows that the biotin added to a yeast sample is quantitatively recovered. Treatment of such samples with potassium permanganate results in a complete inactivation. Oxybiotin, on the other hand, is not significantly inactivated, and the growth-promoting activity of permanganate-treated samples of biological materials appears to be representative of their oxybiotin content.

*Nature of Growth Factor in Organisms Grown in Media Containing Biotin and Oxybiotin*—It was of interest to grow microorganisms in culture media

TABLE I  
*Recovery of Biotin and Oxybiotin Added to Difco Autolyzed Yeast*  
The results are given in millimicrograms.

Biotin content of Difco yeast sample	Growth factor added	Total biotin found	Growth factor found after KMnO <sub>4</sub> treatment	Recovery of added growth factor
				per cent
385	430 <i>d</i> -Biotin	840	0	103
385	1800 <i>dl</i> -Oxybiotin	*	1810	100
485	430 <i>d</i> -Biotin	970	0	106
485	1500 <i>dl</i> -Oxybiotin	*	1400	93

\* Because of the difference in the shapes of the yeast growth curves, an exact determination of biotin in the presence of oxybiotin is not possible.

containing either biotin, oxybiotin, or desthiobiotin, and subsequently to determine the character of the growth factor present within the cells.

*Saccharomyces cerevisiae* was grown in 500 ml. portions of nutrient medium (9) containing the amounts of growth promoter specified in Table II. Following an incubation period of 2 days at 37°, each crop of yeast cells was collected by centrifugation and washed several times by resuspension in distilled water and recentrifugation. The washed yeast cells were suspended in 20 ml. of water, and a 10 ml. aliquot was used for hydrolysis (with 5 ml. of 9 N sulfuric acid). Aliquots of the remaining yeast suspension were dried to constant weight *in vacuo* to determine their dry weight. A yield of approximately 1 gm. of dry yeast was obtained from 500 ml. of medium. In similar experiments, *Rhizobium trifolii* was grown in 500 ml. of medium (11) at 28° for 12 days. The yields were approximately 0.2 gm. of dry cells. The latter were hydrolyzed with proportionally smaller amounts of 3 N sulfuric acid, and assayed by the above method.



Table II summarizes the results which were obtained with the hydrolysates.

It is seen that with varying levels of *d*-biotin in the nutrient medium (Experiments 1 to 3) the growth factor stored in the yeast cells is completely destroyed by the permanganate treatment. The turbidity readings in the bioassays were not significantly different from the blank readings. Similar results were obtained with yeast grown in a medium containing desthiobiotin (Experiment 4).

With yeast grown in the presence of *dl*-oxybiotin (Experiments 5 to 7) the growth-stimulating activity is almost the same for the untreated and for the potassium permanganate-treated hydrolysates.

TABLE II  
*Biotin and Oxybiotin Content of Yeast and Rhizobium Cells Grown in Different Media*

Experiment No.	Organism grown	Growth factor added to 500 ml. medium		Growth factor per gm. dry cells	
				Permanganate treatment omitted	Following permanganate treatment
		$\gamma$		$\gamma$	$\gamma$
1	<i>Saccharomyces cerevisiae</i>	0.5	<i>d</i> -Biotin	0.24	0
2	" "	2.0	"	0.90	0
3	" "	2.5	"	1.15	0
4	" "	5.0	<i>dl</i> -Desthiobiotin	1.5	0
5	" "	5.0	<i>dl</i> -Oxybiotin	2.5	2.4
6	" "	30	"	4.25	3.8
7	" "	0.5	"	0.42	0.42
8	<i>Rhizobium trifolii</i>	1.0	<i>d</i> -Biotin	0.35	0.01
9	" "	5.0	<i>dl</i> -Oxybiotin	0.90	0.80

The results for *Rhizobium trifolii* cells grown with biotin and oxybiotin (Experiments 8 and 9) are similar to those obtained with yeast.

#### DISCUSSION

The finding that permanganate destroys the biotin activity of cells grown in media containing biotin indicates that these cells contain no oxybiotin. In the case of yeast grown with desthiobiotin, there is ample evidence (5, 6) that the cells convert this substance into biotin. Our observations that the growth factor in these cells (grown with desthiobiotin) is destroyed by permanganate agree with previous conclusions.

The fact that the activities of the untreated and permanganate-treated hydrolysates are essentially equal when *Saccharomyces cerevisiae* and

*Rhizobium trifolii* are grown in the presence of oxybiotin indicates that oxybiotin is the factor present within the cells of these microorganisms.

It is evident that the yeast cells can reintroduce the sulfur atom into the desthiobiotin structure. However, the oxybiotin molecule is active as such, without transformation into biotin. These experiments are the first demonstration that the sulfur atom in biotin is not essential for the growth-promoting activity of this compound in *Saccharomyces cerevisiae* and *Rhizobium trifolii*.

Furthermore, it may be pointed out that the biotin-oxybiotin relationship is the first observed instance in which an atom in the ring structure of a natural nitrilite has been replaced without significantly affecting its biological activity.

The authors thank Miss Lola Ward for technical assistance.

#### SUMMARY

A procedure has been developed for the determination of oxybiotin in biological materials. The method is based upon the destruction of biotin by dilute potassium permanganate solution, and the stability of oxybiotin toward this reagent. The yeast growth-stimulating activity of acid hydrolysates of tissues or microorganisms following treatment with permanganate appears to be representative of their oxybiotin content.

This new method has been used to demonstrate that *Saccharomyces cerevisiae* and *Rhizobium trifolii* grown in the presence of oxybiotin utilize this compound as such, and do not convert it into biotin. These results are the first demonstration that the sulfur atom is not essential for the biological activity of biotin.

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# DESTHIOBIOTIN IN THE BIOSYNTHESIS OF BIOTIN\*

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Recent studies on the biotin requirements of a number of microorganisms have shown that they can be divided into two classes with respect to their utilization of desthiobiotin. One group, typified by *Saccharomyces cerevisiae* (1) and including *Neurospora* and certain other fungi (2), needs for growth either desthiobiotin or biotin. The activity of desthiobiotin for *Saccharomyces cerevisiae* has been shown to be associated with its *in vitro* conversion to biotin (3). It seems likely that this conversion occurs in the other organisms of this group.<sup>1</sup> The other group, typified by *Lactobacillus casei* (1) and including certain other bacteria and fungi (2), is unable to grow with desthiobiotin, presumably since this substance cannot be converted to biotin by these organisms. These observations suggest that desthiobiotin is a normal intermediate in the biosynthesis of biotin (4).

The evidence reported in this paper indicates that an x-ray-produced mutant strain of *Penicillium chrysogenum*, strain 62078, synthesizes desthiobiotin but cannot convert it to biotin, and that as a result it requires biotin for growth. In addition, the rôle of pimelic acid in the biosynthesis of biotin (5, 6) has been confirmed by the demonstration that pimelic acid increases the production of desthiobiotin by this strain approximately 10-fold. This evidence strongly supports the hypothesis that desthiobiotin is a normal intermediate in the biosynthesis of biotin.

## EXPERIMENTAL

The following biotin-requiring microorganisms have been used: *Neurospora crassa* 1A, *Escherichia coli*, strain 58 (7), *Penicillium notatum*, strain 21464, and *Penicillium chrysogenum*, strain 62078.<sup>2</sup> *Neurospora* was grown in the medium described by Horowitz and Beadle (8), *Escherichia coli* in the medium given by Gray and Tatum (7), and the *Penicillia* in the Czapek-Dox medium.

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<sup>1</sup> Direct evidence of this conversion has recently been reported by L. H. Leonian and V. G. Lilly (*J. Bact.*, **49**, 291 (1945)).

<sup>2</sup> Bonner, D., Beadle, G. W., and Mitchell, H. K., unpublished.

The specificities of the biotin requirements of these cultures were determined with samples of desthiobiotin, biotin diaminocarboxylic acid, desthiobiotin diaminocarboxylic acid, and biotin sulfone obtained through the courtesy of Dr. Vincent du Vigneaud. The results of these tests showed that *Neurospora crassa* used desthiobiotin for growth just as well as biotin. *Escherichia coli* 58 responded somewhat more slowly to desthiobiotin, but the activities of the two substances were the same after 63 hours incubation. The other compounds had activities for both organisms of less than 0.01

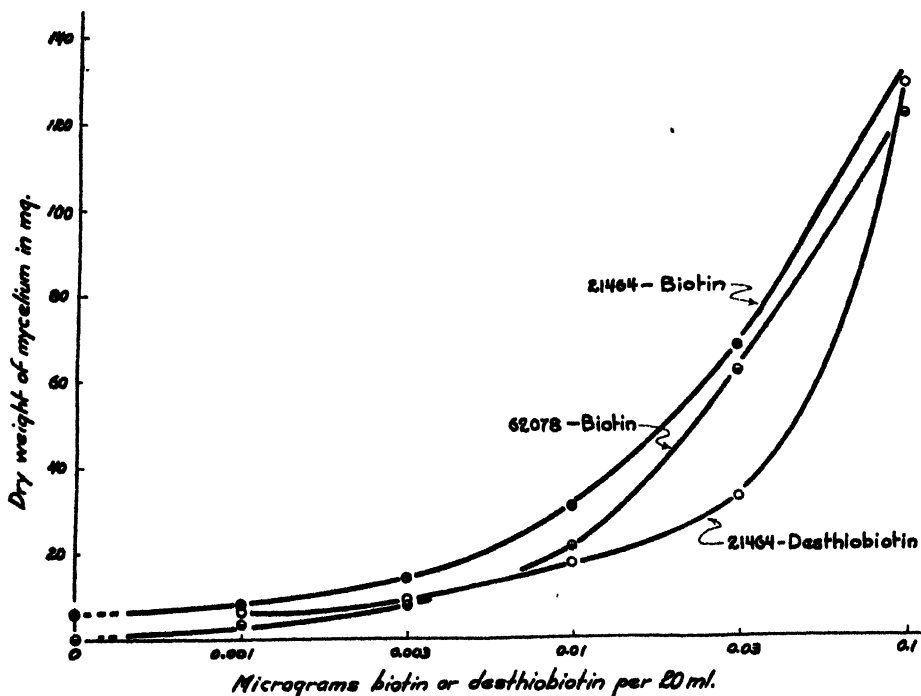


FIG. 1. The responses of *Penicillium* mutant strains to biotin and to desthiobiotin

per cent of that of biotin. Biotin sulfone inhibited the activity of biotin and desthiobiotin for both *Neurospora crassa* and *Escherichia coli* 58, with inhibition ratios (9) of about 1000.

The results of tests of the two biotin-requiring mutant strains of *Penicillia* are given in Fig. 1. Strain 21464 responded to desthiobiotin almost as well as to biotin. In contrast, strain 62078, which has a quantitatively similar biotin requirement, is unable to use desthiobiotin. If these strains of *Penicillium* are analogous to mutant strains of *Neurospora* (10), the reactions which normally lead to the biosynthesis of biotin should be broken in two different single reactions in the two strains, since they

differ in their ability to use desthiobiotin. If strain 62078 owes its need for biotin to the loss of ability to convert desthiobiotin to biotin and if desthiobiotin is a normal intermediate in biotin synthesis by *Penicillium chrysogenum*, this strain should make desthiobiotin. An attempt to demonstrate the production and accumulation of desthiobiotin by this strain was made with culture methods similar to those used in analogous cases with *Neurospora* mutant strains (10, 11).

*Penicillium chrysogenum*, strain 62078, was grown in 125 ml. Erlenmeyer flasks containing 50 ml. of the Czapek-Dox medium supplemented with 0.01 and with 0.1  $\gamma$  of biotin. After 6 days incubation at 25° with constant

TABLE I

*Production of Desthiobiotin by Penicillium chrysogenum 62078 (Assayed after 6 Days Growth at 25° with Constant Shaking)*

Experiment No.	Additions to Czapek-Dox medium, per 50 ml. culture	Desthiobiotin produced, per 50 ml. culture	
		<i>Neurospora crassa</i> assay	<i>Escherichia coli</i> 58 assay
		$\gamma$	$\gamma$
1	0.01 $\gamma$ biotin	0.235	
	0.1 " "	0.235	
2	0.01 " "	0.46	0.41
	0.01 " " + 1 mg. pimelic acid	5.9	5.6
	0.01 " " + 1 " cystine	0.52	0.48
	0.01 " " + 1 " pimelic acid + 1 mg. cystine	5.5	5.8
3	0.01 $\gamma$ biotin	0.23	
	0.01 " " + 1 $\gamma$ pimelic acid	0.25	
	0.01 " " + 10 " " "	0.32	
	0.01 " " + 100 " " "	1.04	
	0.01 " " + 1000 " " "	4.08	
4	0.01 " " + 2000 " " "	3.25*	

\* 0.005  $\gamma$  of biotin by *Lactobacillus casei* assay.

shaking, the cultures were heated to boiling and filtered. The filtrates, assayed for biotin with *Neurospora crassa*, contained approximately 0.23  $\gamma$  of biotin equivalents per 50 ml. of culture (Table I, Experiment 1). These solutions showed biotin activity for *Penicillium* strain 21464 but not for strain 62078, and the failure of strain 62078 to grow was not due to inhibitory substances, since the addition of biotin led to the expected growth. These results suggest that the active substance produced by strain 62078 is not biotin as such.

Further similar experiments were performed, including tests for the effects of pimelic acid and cystine (5, 6) on the production of the biotin-

active substance. The results are given in Table I. Assays for biotin with *Neurospora crassa* and with *Escherichia coli* 58 gave similar values. Pimelic acid increased the amount of active substance from approximately 0.5  $\gamma$  of biotin equivalent to 5.0  $\gamma$  per culture, but cystine had no apparent effect either with or without pimelic acid (Experiment 2). In Experiment 3, the addition of 1 mg. of pimelic acid per 50 ml. of culture was found to give the maximum effect. Pimelic acid with or without cystine had no biotin activity for *Neurospora*, *Escherichia coli* 58, or for the two *Penicillium* strains.

This evidence suggests that the biotin-active substance produced by *Penicillium chrysogenum*, strain 62078, is desthiobiotin. However, since *Lactobacillus casei* is known not to respond to desthiobiotin (1), it was felt that a direct test with this organism was desirable as confirmatory evidence. The filtrate was slightly active on *L. casei* in the medium of Hutchings and Peterson (12). However, on the basis of a curve with known biotin supplements in medium containing additions of a *Penicillium* filtrate which by *Neurospora crassa* assay contained 3.25  $\gamma$  of biotin equivalent per 50 ml. (Experiment 4), the amount of biotin from the *L. casei* assay could not have been over 0.005  $\gamma$  per 50 ml. This result substantiates the conclusion that the biotin-active substance produced by *Penicillium chrysogenum* 62078 is desthiobiotin.

#### DISCUSSION

The results reported in this paper support the conclusion that desthiobiotin is a normal intermediate in the synthesis of biotin by *Penicillium*. In *Penicillium chrysogenum* 62078 pimelic acid seems to be a precursor of desthiobiotin, since its addition resulted in a 10-fold increase in the desthiobiotin produced. This is comparable to the 16-fold increase in the biotin production of *Aspergillus niger* demonstrated by Eakin and Eakin (6), but is a much greater absolute increase in terms of biotin equivalents. Pimelic acid increased the production of biotin by *Aspergillus niger* from 0.006 to 0.1  $\gamma$  per 12 ml., but increased the production of desthiobiotin by *Penicillium chrysogenum* 62078 from 0.5 to 5.0  $\gamma$  per 50 ml. Since cystine had no effect on the synthesis of desthiobiotin, which differs from biotin only in the absence of the sulfur atom in the molecule, it seems likely that cystine is not involved in the synthesis of desthiobiotin, but rather in the conversion of desthiobiotin to biotin.

In view of the effects of x-ray and ultraviolet irradiation in producing biotin-requiring strains of *Escherichia coli* (7) and *Penicillium*,<sup>2</sup> presumably as the result of gene mutations, the biotin requirements of other microorganisms found in nature may have resulted from analogous mutations affecting different biochemical steps in this synthesis. In organisms found in nature, more than one reaction may be blocked as the result of accumu-

lated independent mutations. It also seems likely that the reactions involved in the synthesis of biotin are the same in all microorganisms. If this is true, biotin-requiring microorganisms may be classified into several types, depending on the location of the terminal block in the synthesis in each case.

In one class, represented by the diphtheria bacillus (3), the synthesis of pimelic acid is blocked. In another class the synthesis of desthiobiotin from pimelic acid is blocked. This class may include *Saccharomyces cerevisiae* (1), *Ceratostomella ips* 438, *Neurospora crassa* and *N. sitophila* (2, 13), *Penicillium notatum* 21464, and *Escherichia coli* 58, since these can use desthiobiotin but not pimelic acid. A third class including *Lactobacillus casei* (1), *Ceratostomella pini* 416, *Sordaria fimicola*, *Lactobacillus arabinosus*, *Rhizobium trifolii* 205 (2), and *Penicillium chrysogenum* 62078 would consist of those organisms which cannot convert desthiobiotin to biotin.

#### SUMMARY

*Neurospora crassa*, *Escherichia coli* 58, and *Penicillium notatum* 21464 grow as well on desthiobiotin as on biotin, but *Penicillium chrysogenum* 62078 is unable to grow on desthiobiotin.

The production of a substance with the specific biological activity of desthiobiotin by *Penicillium chrysogenum* 62078 has been demonstrated. The addition of pimelic acid increased the production of this substance from 0.5  $\gamma$  to 5.0  $\gamma$  of biotin equivalent per 50 ml. of culture. Cystine had no effect.

These observations suggest that desthiobiotin is a normal intermediate in the biosynthesis of biotin by *Penicillium chrysogenum*, and probably also in other microorganisms.

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# THE FUNCTION OF PYRIDOXINE: CONVERSION OF MEMBERS OF THE VITAMIN B<sub>6</sub> GROUP INTO CODECARBOXYLASE

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Snell and coworkers (1-4) have recently demonstrated that three compounds, pyridoxine, pyridoxamine, and pyridoxal,<sup>1</sup> show vitamin B<sub>6</sub> activity for various organisms. All three compounds are utilized by *Saccharomyces carlsbergensis*, *Neurospora sitophila*, and the rat, pyridoxal and pyridoxamine are effective for *Streptococcus faecalis* R, while *Lactobacillus casei* responds only to pyridoxal (4). The wide distribution of these three compounds in nature has also been established (5).

Studies on the function of the vitamin B<sub>6</sub> group have shown that pyridoxal can be converted biologically and chemically into a compound of unknown structure which serves as the coenzyme of amino acid decarboxylases (6). Gale and Epps (7) have isolated a substance from natural products which also serves as the coenzyme of amino acid decarboxylases, and have termed this material *codecarboxylase*. As other studies (8) have shown the similarity of the natural preparations to those prepared biologically and chemically from pyridoxal, the term *codecarboxylase* is used in this paper to refer to all preparations which possess coenzyme activity for the amino acid decarboxylases. To date four amino acid decarboxylases, tyrosine (7, 9), lysine (7), arginine (10), and glutamic acid (10), have been shown to require this coenzyme.

The present work<sup>2</sup> was undertaken to determine whether members of the vitamin B<sub>6</sub> group in addition to pyridoxal are converted into *codecarboxylase*. For this purpose, various organisms were grown in media with pyridoxine, pyridoxamine, or pyridoxal as the source of vitamin B<sub>6</sub> and the cells harvested and assayed for *codecarboxylase*. It has been found that the three known members of the vitamin B<sub>6</sub> group are converted, at least partially, into *codecarboxylase* by growing cultures. In addition, resting cells of *Streptococcus faecalis* will convert pyridoxamine into *codecarboxylase* if pyruvate is also present.

A correlation has been found between the *codecarboxylase* content of rat tissue and the pyridoxine content of the diet.

<sup>1</sup> We wish to thank the Research Laboratories of Merck and Company, Inc., for the pyridoxal and pyridoxamine used in these studies.

<sup>2</sup> A preliminary report of this work was given at the spring meeting of the Central New York Branch of the Society of American Bacteriologists (*J. Bact.*, in press).

More codecarboxylase activity is obtained per mole of pyridoxal, or of other members of the vitamin B<sub>6</sub> group, furnished during growth than by the addition of pyridoxal to resting cells, or cell preparations, of *Streptococcus faecalis* R.

### Methods

Both growing cultures and resting cell suspensions have been tested for their ability to convert members of the vitamin B<sub>6</sub> group into codecarboxylase. Growing cultures would appear to offer the greater promise for the formation of codecarboxylase, because a culture which can use a vitamin to fulfil its growth requirement would seem of necessity to convert that vitamin into a functional unit. On the other hand, resting cells would be more specific in their requirement and, therefore, might be expected to yield more information as to the mechanism of coenzyme formation.

Two methods have been employed to detect codecarboxylase. The first, which was used with *Streptococcus faecalis* R, depends on the formation of tyrosine decarboxylase apoenzyme by this culture when it is grown in the absence of members of the vitamin B<sub>6</sub> group (11). Cells from such cultures do not decarboxylate tyrosine unless substances are furnished, either during growth or to the resting cells after harvest, which are converted into codecarboxylase. As the rate of tyrosine decarboxylation by these cells has been shown to be a function of the coenzyme concentration, the formation of codecarboxylase can be determined directly (8).

The second method used for the determination of codecarboxylase depends on the extraction of the coenzyme from cells grown with various members of the vitamin B<sub>6</sub> group and the assay of the extract with the tyrosine decarboxylase apoenzyme of *Streptococcus faecalis* R (8). This procedure was, of necessity, applied to those cultures for which specific amino acid decarboxylases were unknown. Because pyridoxal was the only substance available in pure form, which activates the decarboxylase system, the assay was standardized in terms of pyridoxal (plus adenosine triphosphate) and the amount of coenzyme expressed in terms of "pyridoxal equivalents." The extraction procedure, which was adapted from the studies of Gale and Epps (7), was as follows: The tissues were homogenized in 0.1 N NaOH, heated to 100° for 5 minutes, cooled, and adjusted to pH 5.0. Aliquots were assayed without removing the suspended matter.

### EXPERIMENTAL

*Codecarboxylase Production by Streptococcus faecalis*—A cell suspension of *Streptococcus faecalis* grown in a vitamin B<sub>6</sub>-deficient medium (9) will convert pyridoxal into codecarboxylase, and dried cell preparations will also convert pyridoxal into this coenzyme if adenosine triphosphate is furnished

(6). The quantitative response of the preparations to the addition of pyridoxal is shown in Table I. As the optimum pH for decarboxylation by living cells is slightly lower than that for cell preparations, each was run at its optimum pH. The level of pyridoxal required for half maximum stimulation of the apoenzyme is similar with the resting cells and the dried preparation. From the amount required, about 0.15  $\gamma$  per 3 ml. of fluid volume, the dissociation constant has been calculated as  $3 \times 10^{-7}$  mole per liter (8, 9).

To compare the conversion of pyridoxal into codecarboxylase by growing cells with that formed by resting cells, or cell preparations, *Streptococcus*

TABLE I

*Codecarboxylase Formation from Pyridoxal by Cell Suspensions and Cell Preparations of Streptococcus faecalis R*

Per Warburg cup, in the side arm, 0.5 ml. of M/30 tyrosine suspension; in the cup, *cell suspension*, cells from 3 ml. of medium  $\approx$  0.25 mg. of bacterial N, 1 ml. of M/15 phthalate buffer (pH 5.0), and water or pyridoxal to 2.5 ml.; *cell preparation*, 1 mg. of dried cell preparation,\* 1 ml. of 0.2 M acetate buffer (pH 5.5), 1 mg. of adenosine triphosphate, and water or pyridoxal to 2.5 ml.

Pyridoxal added $\gamma$ per 3 ml.	CO <sub>2</sub> evolved	
	Cell suspension microliters per hr	Cell preparation* microliters per hr.
0.0	3	15
0.01		38
0.04	57	62
0.06	78	80
0.3	177	283
0.6	228	343
10.0	400	440

\* The dried cell preparation may also be used for the assay of coenzyme, in which case the adenosine triphosphate is omitted (8).

*faecalis R* was grown with graded amounts of pyridoxal. It was felt that this would afford a method for the standardization of an extraction procedure for the coenzyme. That is, the rate of tyrosine decarboxylation by cells grown with different levels of pyridoxal could be determined directly, and the codecarboxylase content calculated by comparison to the CO<sub>2</sub> evolution obtained with the assay preparation in the presence of an aliquot of the cell extract. To determine the codecarboxylase content from the rate of tyrosine decarboxylation the total amount of enzyme per Warburg cup would need to be held constant. Cells grown with 0.03  $\gamma$  or less of pyridoxal per 3 ml. contained similar amounts of tyrosine decarboxylase

(Table II, third column); so that within these limits the rate of tyrosine decarboxylation can be compared to the rate observed with the assay system (Fig. 1). As may be seen from the curves, the cells grown with pyridoxal gave a greater rate of decarboxylation per unit of pyridoxal furnished than did the resting cell suspension to which pyridoxal was added after harvest. About 10 times as much pyridoxal are required for a given rate of decarboxylation by the resting cells as by the growing culture. The present data are not sufficient to determine whether the greater activity of

TABLE II  
*Codecarboxylase Formation from Pyridoxal by Growing Cultures of Streptococcus faecalis*

Per Warburg cup, in the side arm, 0.5 ml. of M/30 tyrosine suspension; in the cup, cells from 3 ml. of medium (0.25 mg. of bacterial N) and 1.0 ml. of M/15 phthalate buffer (pH 5.0).

Pyridoxal in medium	CO <sub>2</sub> evolved by cells from 3 ml. of medium		Codecarboxylase assay of cell extract	
	As harvested	+ 10 $\gamma$ pyridoxal	Pyridoxal equivalent	Activity*
$\gamma$ per 3 ml.	microliters per hr.	microliters per hr.	$\gamma$ per 3 ml.	
0.0	3	450	0.0045	
0.0015	16	360	0.006	4.0
0.003	40	150	0.054	18.0
0.0045	55		0.055	12.2
0.006	64	450	0.072	12.1
0.012	87		0.078	6.5
0.03	120	400	0.084	2.8
0.09	142	200†	0.240	2.6
0.9	190	200†	0.700	0.8

\* Activity = micrograms of codecarboxylase (pyridoxal equivalents) per microgram of pyridoxal furnished to the growing culture.

† Indicates that cells have a lower enzyme content. It appears that the decreased enzyme content at the higher levels of pyridoxal is due to the exhaustion of the tyrosine from the medium. The addition of tyrosine to the medium increased the apoenzyme content in these cases.

the cells grown with pyridoxal indicates a more efficient conversion of pyridoxal to coenzyme or the formation of a different type of coenzyme. For the present it is assumed that the greater activity observed when pyridoxal is present during growth is due to more efficient conversion by growing cultures.

The increased activity observed in cells grown in the presence of pyridoxal does not allow a determination of the exact degree of conversion of vitamin B<sub>6</sub> derivatives to codecarboxylase. As mentioned above, the cells grown with pyridoxal were extracted and assayed for codecarboxylase with the

dried cell preparation. The assay data, given in Table II, also indicate that the growing culture is 15 to 20 times as efficient in converting pyridoxal into codecarboxylase as is the assay system, thus affording satisfactory agreement with the data obtained with living cell suspensions (Fig. 1). As may be seen from Table II, those cells grown without pyridoxal show a small amount of codecarboxylase by the extraction procedure.

Calculations of the efficiency of codecarboxylase production from other members of the vitamin B<sub>6</sub> group must be considered in the light of this

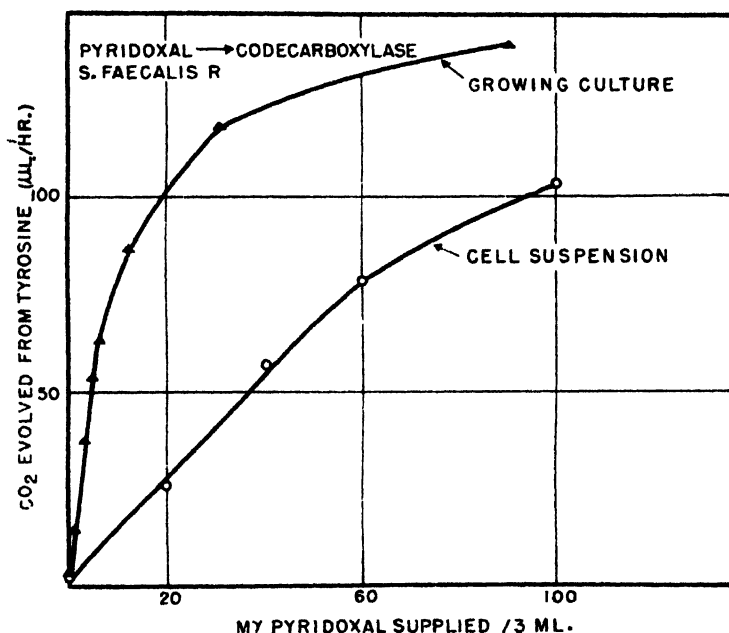


FIG. 1. Codecarboxylase activity of cells grown with pyridoxal *versus* cells grown without and furnished pyridoxal in Warburg cups. Per cup, in the side arm, 0.5 ml. of M/30 tyrosine; in the main cup, 1 ml. of M/15 phthalate buffer (pH 5.0), cells from 3 ml. of medium (0.25 mg. of bacterial N), and pyridoxal or water to 2.5 ml.; 28°.

finding. That is, recoveries of 1000 to 2000 per cent based on the amount of vitamin B<sub>6</sub> member furnished may be expected when the coenzyme activity is expressed in terms of pyridoxal equivalents.

*Streptococcus faecalis*, strain R, as shown by Snell (1), is able to utilize pyridoxal and pyridoxamine for growth. The data of Table III show that pyridoxamine as well as pyridoxal is converted into codecarboxylase by growing cultures, and that codecarboxylase recoveries as high as 13 times the pyridoxamine furnished were obtained.

Resting cell suspensions of *Streptococcus faecalis* R do not convert pyri-

doxamine to codecarboxylase (9) in the tyrosine system at pH 5.0. Such suspensions, however, will convert pyridoxamine into codecarboxylase if a keto acid, such as pyruvate, is added and the mixture (cells, pyridoxamine,

TABLE III

Conversion of Pyridoxamine to Codecarboxylase by Growing Cultures of *Streptococcus faecalis* R

Pyridoxamine supplied $\gamma$ per 10 ml.	Tyrosine decarboxylation by cell suspensions $CO_2$ (N)	Codecarboxylase assay of cell extract	
		Pyridoxal equivalent $\gamma$ per 10 ml.	Activity*
0.0	3	0.004	
0.001	32	0.017	13
0.010	62	0.040	4
0.040	362	0.036	0.8

\* Micrograms of codecarboxylase per microgram of pyridoxamine furnished.

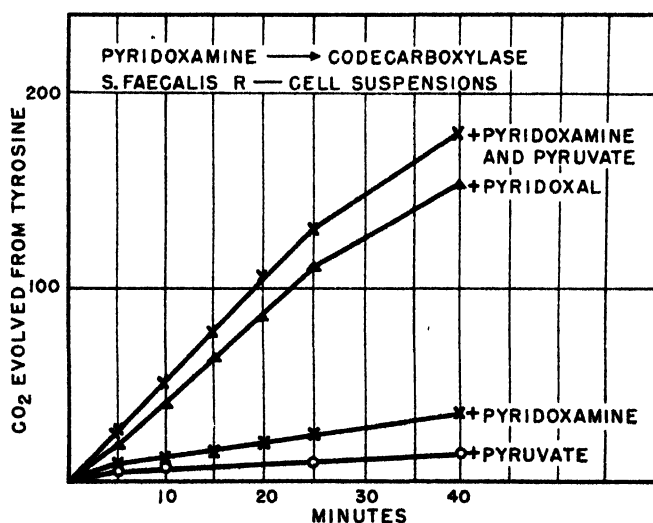


FIG. 2. Conversion of pyridoxamine to codecarboxylase by cell suspensions. The cells were incubated for 30 minutes at 28° with 3  $\gamma$  of pyridoxamine, 3 micromoles of pyruvate, or both, in 2 ml. of M/300 phosphate buffer, pH 7.0, and then added to a Warburg cup which contained, in the side arm, 0.5 ml. of M/30 tyrosine, in the main cup, 1.0 ml. of M/15 phthalate buffer, pH 4.8 (final pH 5.0). The  $CO_2$  evolved from tyrosine is measured in microliters per hour.

and pyruvate) incubated at pH 7 before the tyrosine decarboxylation is determined at pH 5 (Fig. 2). The incubation at pH 7 is necessary; cells incubated with pyridoxamine and pyruvate at pH 5 do not form code-

carboxylase. The formation of codecarboxylase from pyridoxamine in the presence of pyruvate would appear to involve transamination in which the amino group of pyridoxamine is transferred to pyruvate to yield pyridoxal and alanine, the pyridoxal being converted into codecarboxylase. This reaction would constitute the biological counterpart of the chemical transformation of pyridoxamine into pyridoxal by heating with keto acids (Snell (12)).

*Codecarboxylase Production by Saccharomyces carlsbergensis*—*Saccharomyces carlsbergensis* is able to utilize pyridoxine, pyridoxamine, and pyridoxal equally well for growth (4). To test the ability of this culture to

TABLE IV  
Conversion of Members of Vitamin B<sub>6</sub> Group to Codecarboxylase by  
*Saccharomyces carlsbergensis*

Growth medium, base medium (9) plus 50  $\gamma$  of thiamine and 5 mg. of inositol per 100 ml. of medium.

	Amount supplied	Growth	Codecarboxylase in cell extract by assay*	Micrograms codecarboxylase Micrograms vitamin B <sub>6</sub> furnished
	$\gamma$ per 100 ml.	turbidity†	$\gamma$ per 100 ml.	
Pyridoxal	0.0	8	0.15	
	0.05	10	0.25	5
	0.1	10	0.25	2.5
Pyridoxamine	0.5	30	2.44	4.9
	0.05	12	0.52	10
	0.1	30	1.70	17
Pyridoxine	0.5	150	2.03	4.1
	0.05	12	0.93	19
	0.1	16	1.73	17
	0.5	145	2.44	4.9

\* Extraction and assay described in the text.

† Each scale unit  $\cong$  6  $\gamma$  of bacterial N per 10 ml

convert members of the vitamin B<sub>6</sub> group into codecarboxylase the organism was inoculated into 50 ml. quantities of broth in 125 ml. Erlenmeyer flasks and incubated with shaking for 18 hours at 30°. The cells were harvested, extracted, and assayed for codecarboxylase. The results (Table IV) show that the three members of the vitamin B<sub>6</sub> group are converted into codecarboxylase and the activity per mole of vitamin B<sub>6</sub> furnished is 10 to 20 times that obtained with the pyridoxal-adenosine triphosphate standard.

*Codecarboxylase Production by Neurospora sitophila*—The *pyridoxineless* x-ray mutant of *Neurospora sitophila* (Beadle and Tatum (13)) may be used for the assay of pyridoxine (14). This strain responds equally well to all



three members of the vitamin B<sub>6</sub> group (4). Stokes *et al.* (14) have shown that the culture will grow without pyridoxine at a pH above 5.8 and will synthesize pyridoxine, as indicated by the *Neurospora sitophila* and *Lactobacillus casei* assay methods. The data presented in Table V show that codecarboxylase is formed from the three members of the vitamin B<sub>6</sub> group. With this culture also the conversion factor is 10 or more. The value of 43 for a low level of pyridoxal is the highest so far obtained. It should be noted that in the absence of the vitamin B<sub>6</sub> group this culture grows slightly and contains material which activates the assay system; thus the factor of 43 may be higher than actually occurs.

TABLE V  
*Conversion of Members of Vitamin B<sub>6</sub> Group into Codecarboxylase by Neurospora sitophila*<sup>a</sup>

Growth medium, base medium (9) adjusted to pH 5.0 after autoclaving.

	Amount supplied	Growth (cell nitrogen)	Codecarboxylase in cell extract by assay†	Micrograms codecarboxylase Micrograms vitamin B <sub>6</sub> furnished
	$\gamma$ per 25 ml.	mc per 25 ml		
Pyridoxal	0.0	0.6	0.12	
	0.01	1.5	0.55	43
	0.04	14	0.72	15
	1.5	19	0.7	5
Pyridoxamine	0.04		0.56	11
Pyridoxine	0.04		0.42	7.5

<sup>a</sup> We wish to thank Dr. Beadle for supplying this culture.

† Extraction and assay described in the text.

*Neurospora sitophila*, when grown under conditions permitting synthesis of vitamin B<sub>6</sub> as described by Stokes *et al.* (14), forms codecarboxylase (Table VI).

Table VI also shows that several other organisms which do not require vitamin B<sub>6</sub> for growth form codecarboxylase in considerable quantities. One culture, *Streptococcus lactis* L103, yielded only a small amount of coenzyme.

*Relation between Pyridoxine Fed and Codecarboxylase in Rat Tissue*<sup>a</sup>—Through the facilities of the Merck Institute for Therapeutic Research we were provided with tissues from rats on diets deficient in vitamin B<sub>6</sub> derivatives and with those from animals on identical diets supplemented with pyridoxine. After the growth experiments, portions of muscle and of liver

<sup>a</sup> We wish to thank the Merck Institute for Therapeutic Research for the rat tissues.

TABLE VI

*Presence of Codecarboxylase in Organisms Grown on Medium Deficient in Vitamin B<sub>6</sub> Group*

Growth medium, base medium (9).

Organism	Growth temperature	Age of culture	Volume of medium	Codecarboxylase assay of cell extracts (pyridoxal equivalents)
	°C.	hrs	ml.	$\gamma$ per 100 ml.
<i>Escherichia coli</i> , Crookes strain*	37	19	30	1.30
" " 86	28	19	30	1.87
" " 86	37	19	20	0.32
<i>Streptococcus lactis</i> L103	28	19	175	0.01
<i>Lactobacillus arabinosus</i> 17-5	35	24	100	0.155
<i>Neurospora sitophila</i> , pyridoxineless, pH 7.0	25	72	25	1.6

\* Arginine and glutamic acid decarboxylases are present, for which codecarboxylase is the coenzyme (10).

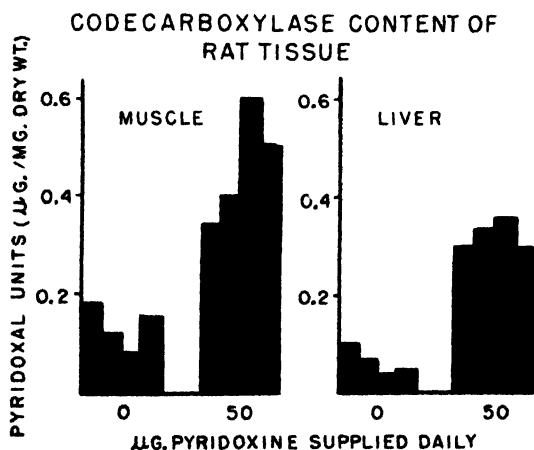


FIG. 3. Influence of pyridoxine in the diet on the codecarboxylase content of rat tissue. The tissues were extracted with alkali and the extract assayed with tyrosine decarboxylase apoenzyme.

were removed, frozen, and shipped to our laboratories in dry ice. Upon arrival, the frozen tissue was extracted with alkali as previously described and the extract assayed for codecarboxylase.

Two series of animals were analyzed. In the first, the codecarboxylase content of muscle and liver from the deficient rats was compared with the

level found in animals on the identical diet supplemented with 50  $\gamma$  of pyridoxine daily. The data (Fig. 3) show that the tissues from the animals which received pyridoxine contained several times as much codecarboxylase as those from the animals on the deficient diet.

In the second experiment, tissues of rats which received graded levels of pyridoxine were analyzed. The data (Fig. 4) show that, while some variation in codecarboxylase content occurs, there is a relationship between the pyridoxine fed and the codecarboxylase content of the muscle. The points connected by the line represent the average codecarboxylase content of each group of animals. If these are plotted to scale, a straight line relationship between pyridoxine supplied and the codecarboxylase content of the tissue is found. The growth requirement was met by the 15  $\gamma$  level

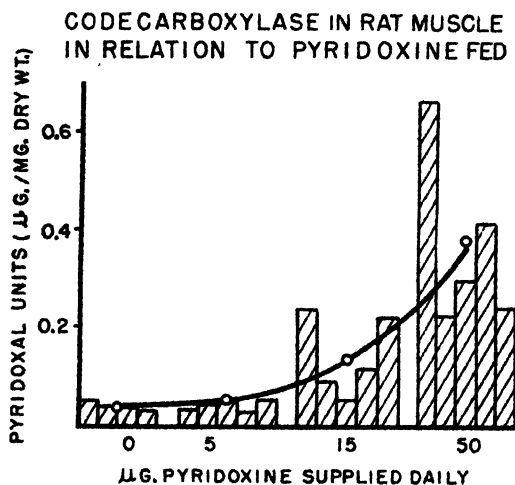


Fig. 4. Codecarboxylase content of tissue from rats on varied levels of pyridoxine

of pyridoxine, but the analyses (Fig. 4) show that an increased supply of pyridoxine, beyond that required by growth, yielded a higher level of codecarboxylase.

#### DISCUSSION

The finding that the three known members of the vitamin B<sub>6</sub> group, pyridoxine, pyridoxamine, and pyridoxal, are converted into codecarboxylase by the organisms capable of utilizing them for growth would support the conception that codecarboxylase is an important functional form of vitamin B<sub>6</sub>. To date, codecarboxylase is known to function as the coenzyme of four amino acid decarboxylases. The relationship of pyridoxine to protein, or amino acid metabolism (15-17), may involve the conversion

of pyridoxine into codecarboxylase by the animal. The data presented here for rat tissues would favor this view.

In addition to the direct conversion of pyridoxal to codecarboxylase, activation of pyridoxamine as a precursor of codecarboxylase by pyruvate suggests that the pathway of formation of the codecarboxylase is through pyridoxal. This formation of the coenzyme from pyridoxamine is most readily interpreted as the transamination of pyridoxamine and pyruvate to pyridoxal and alanine, a process which may also be related to the replacement of pyridoxine derivatives by alanine for the growth of this culture. The observation that alanine may substitute for vitamin B<sub>6</sub> for the growth of *Streptococcus faecalis* R has been interpreted as an indication of the synthesis of vitamin B<sub>6</sub> from alanine (18, 19). However, as has previously been shown (11), alanine does not serve as a precursor of codecarboxylase for *Streptococcus faecalis* R, as indicated by the fact that cells grown with alanine, instead of members of the vitamin B<sub>6</sub> group, yield the tyrosine apoenzyme and not the complete enzyme as would be expected if codecarboxylase were synthesized.

#### SUMMARY

The known members of the vitamin B<sub>6</sub> group are converted into codecarboxylase by microorganisms capable of utilizing them as a source of vitamin B<sub>6</sub>.

The efficiency of codecarboxylase formation by growing cells is from 10 to 20 times as great as with cell suspensions or cell preparations of *Streptococcus faecalis* R.

Cell suspensions of *Streptococcus faecalis* R form codecarboxylase from pyridoxamine in the presence of a keto acid.

Organisms which are capable of growing without vitamin B<sub>6</sub> have been shown to synthesize codecarboxylase.

The codecarboxylase content of rat tissue is directly related to the pyridoxine level in the diet.

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# THE SYNTHESIS AND CONFIGURATION OF *d*-14-METHYLPALMITIC ACID AND ITS IDENTITY WITH THE NATURAL ACID FROM WOOL FAT

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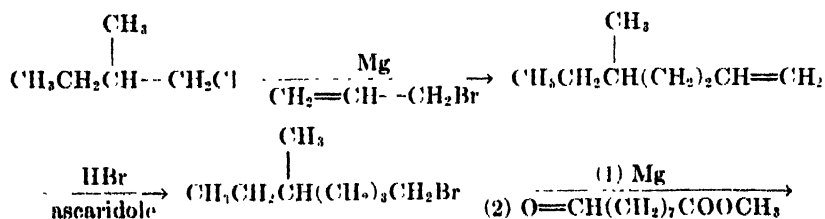
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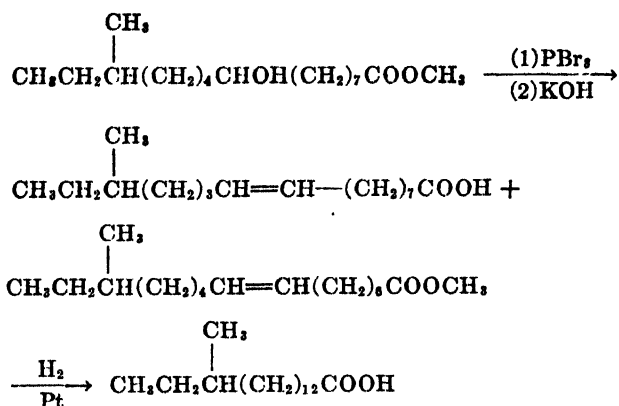
The acidic components of wool fat have recently been resolved by Weitkamp into four homologous series of acids (1). One of these, the so called *anteiso* series, consists of optically active members with an odd number of carbon atoms. The proposed structure of the anteiso acids was deduced from binary melting point curves and from certain geometric and crystallographic considerations. It was concluded that the acids contained even numbered chains of carbon atoms and a methyl group in the antepenultimate position. Credence was lent this view by the correspondence in properties of the C<sub>19</sub> member with synthetic *dl*-16-methylstearic acid of which it was believed to be the optically active form. The direction and magnitude of the optical rotation indicated, according to the rule of Boys (2), that the configuration of the active center was the same as that of *d*(-)-2-methylbutanol-1 from fusel oil.

The proposed structure of the anteiso acids, although without precedent among the known higher acids of mammalian fats, is formally similar to that of tuberculostearic acid isolated from the lipids of the tubercle bacillus (3-5) and to phytomonie acid isolated from the crown gall bacillus, *Phytoplasma tumefaciens* (6). We therefore considered it desirable, especially in view of the unusual method of structure determination employed by Weitkamp, to establish the structure and configuration by synthesis, in the optically active form, of one member of the series. The acid selected was *d*-14-methylpalmitic acid and the starting material *d*(-)-2-methylbutanol-1, obtained by fractional distillation from fusel oil.<sup>1</sup>

The following scheme was employed in the synthesis.



<sup>1</sup> We are indebted to Dr. Philip G. Stevens and Dr. Frank C. Whitmore for half of the active amyl alcohol used in this work, and to Dr. Stevens for the loan of the Stedman column.



The carbon chain of active amyl alcohol was first lengthened by 3 carbon atoms by coupling it through its Grignard reagent with allyl bromide. This was done, with slight loss in activity, in order to remove the active center from the site of subsequent reactions which otherwise might be expected to cause more extensive racemization. Hydrogen bromide was then added to the resulting 5-methylheptene-1 in the presence of ascaridole (7). The product consisted largely of 1-bromo-5-methylheptane which was freed of secondary bromides by fractional distillation. The procedure in the subsequent steps was essentially that employed by Tomecko and Adams in the synthesis of long chain hydroxy acids (8). The 1-bromo-5-methylheptane was converted to the Grignard reagent and condensed with 1 equivalent of 8-carbomethoxyoctanal-1 obtained by ozonization of methyl oleate. Dehydration of the resulting 9-hydroxy-14-methylpalmitic acid was achieved by replacement of the hydroxyl group with bromine and subsequent removal of hydrogen bromide by the action of alcoholic potassium hydroxide. The unsaturated product was hydrogenated and the resulting *d*-14-methylpalmitic acid purified by fractional distillation of its methyl ester.

The properties of the natural and synthetic acids are listed in Table I. Although slight discrepancies are observed, there is essential agreement throughout. The slightly low melting points of the synthetic acid and its amide are to be attributed to a small amount of racemization incurred during the synthesis and to the possible presence of about 3 per cent of inactive isoamyl alcohol in the starting material. The mixed melting points, with samples kindly provided by Dr. Weitkamp, showed no significant depression and the long crystal spacings of the amides, determined by x-ray diffraction, were the same. It may be concluded therefore that the structure and configuration proposed by Weitkamp are correct.

Since the configuration of the anteiso acids is the same as that of *d*(-)-2-

methylbutanol-1, it is also identical with that of *l*(-)-isoleucine. This relationship suggests that the biosynthesis of the anteiso acids may begin with isoleucine. Similarly the so called *iso* series of acids from wool fat, with a terminal isopropyl group, may be built up from valine or its derivatives such as isovaleraldehyde or isovaleric acid, which is known to occur in nature in the oil of the porpoise, *Delphinium globiceps* (10). The addition of 2-carbon units at the carboxyl ends would produce the desired series with even and odd chains respectively. However, it is difficult by a similar analogy from known intermediates to suggest an origin for tuberculostearic and phytomonic acids with secondary methyl groups near the middles of the chains.

TABLE I

*Comparison of the Natural and Synthetic d-14-Methylpalmitic Acids and Their Derivatives*

	Acid		Amide		Methyl ester B.p.
	M.p.*	Rotation, $\alpha_D$	M.p.	Long crystal spacing	
	°C.		°C	Å	°C
Synthetic	36 -36.8	+5.16	88 -89.5	32.8†	156° at 1.8 mm.
Natural	37.3-37.6	+5.23	90.6	32.8†	156° " 1.9 "
Mixed	37 -37.4		89.4-89.8		

\* These were points of complete melting in a capillary in a stirred, electrically heated bath (9). The value for the natural product was redetermined by this method for purposes of comparison with the synthetic material. Weitkamp reported 36.8° for natural acid

† Possible error  $\pm 0.3$  Å.

It is apparent that natural fats have never been examined by methods as powerful as those employed by Weitkamp, and the question is raised whether the new acids are not more widely distributed in nature. In this respect it should be emphasized that the new acids are not combined with sterols as stated by Weitkamp, but with complex triterpenoid alcohols which are not found in ordinary mammalian fats (11). It is possible that related acids may be found in the lipids associated with the hair of other species than sheep.

#### EXPERIMENTAL

*d*(-)-2-Methylbutanol-1—Reagent grade isoamyl alcohol (Merck),  $\alpha_D^{25} = -0.9^\circ$  in substance in a 1 dm. tube, was fractionally distilled three times through a Stedman column containing 4 ft. of packed section. The observed rotation of the constant boiling fraction was  $\alpha_D^{25} = -4.66^\circ$ .



Brauns reported  $\alpha_D^{20} = -4.71^\circ$  for a carefully purified sample (12) and Whitmore and Olewine an average value of  $\alpha_D^{27.5} = -4.77^\circ$  for material distilled through the 38 ft. packed column at Pennsylvania State College (13). The latter authors believed their material to be 95 per cent pure or better.

*d-1-Chloro-2-methylbutane*—This compound was prepared from the alcohol by the action of thionyl chloride and pyridine according to the procedure of Clarke and Streight (14). From 50 gm. of alcohol there were obtained 41.7 gm. of chloride (70 per cent of theory), b.p.  $99-100^\circ$ ,  $n_D^{22} 1.4111$ .

*Optical Rotation*—In homogeneous substance in a 1 dm. tube,  $d_4^{20} 0.8857$ ,  $\alpha_D^{20} = +1.43^\circ$ ,  $[\alpha]_D^{20} = +1.62^\circ$ . Brauns reported  $[\alpha]_D^{20} = +1.644^\circ$  for the chloride (12) and Whitmore and Olewine  $[\alpha]_D^{25} = +1.66^\circ$  (13).

*d-5-Methylheptene-1*—The Grignard reagent, prepared from 33 gm. of *d-1-chloro-2-methylbutane*, 20 gm. of magnesium turnings, and 1 cc. of methyl iodide in 200 cc. of ether, was siphoned under dry nitrogen from the excess magnesium into a 3-necked flask fitted with a sealed stirrer, dropping funnel, and efficient condenser. 120 gm. (3 moles) of allyl bromide were gradually added through the dropping funnel. The reaction began spontaneously after a short induction period and continued with vigor. When it had subsided the mixture was refluxed for 30 minutes and allowed to stand overnight. The product was decomposed with aqueous ammonium chloride, washed, and distilled through a Widmer column. The fraction boiling above  $80^\circ$  was shaken with aqueous silver nitrate for 15 minutes to remove traces of allyl iodide and was then made alkaline, steam-distilled, and finally dried over calcium chloride. On redistillation from potassium carbonate through a 1 ft. Podbelniak column there were obtained 22.6 gm. (65 per cent of theory) of 5-methylheptene-1,  $n_D^{25} = 1.4076$ , b.p.  $113^\circ$ .

$C_8H_{16}$  (112). Calculated, C 85.7, H 14.3; found C 85.2, H 14.7

*Optical Rotation*—In homogeneous substance in a 1 dm. tube,  $\alpha_D^{26} = +7.28^\circ$ ,  $d_4^{26} 0.7124$ ,  $[\alpha]_D^{26} = +10.2^\circ$ .

*d-1-Bromo-5-methylbutane*—A redistilled commercial sample of octene-1 gave good yields of primary bromide when treated with hydrogen bromide in the presence of benzoyl peroxide. However, under the same conditions *d-5-methylheptene-1* yielded a product which contained much labile secondary bromide and showed too high a rotation,  $\alpha_D^{25} = +11.5^\circ$  in substance in a 1 dm. tube. It was subsequently found with freshly synthesized heptene-1 that benzoyl peroxide, due presumably to its low solubility, was virtually inactive in promoting abnormal addition and that the above erratic results were due to the presence of natural peroxides in the octene-1 used. Ascaridole as recommended by Kharasch and co-workers (7) was therefore employed.

25 gm. of 5-methylheptene-1 and 1.5 gm. of ascaridole in 50 cc. of pentane were chilled to  $-70^{\circ}$  in an acetone-dry ice bath. Hydrogen bromide, generated from tetralin and bromine and purified in a red phosphorus and calcium chloride train, was passed into the chilled solution until 25 gm. had condensed (theory 15 gm.). The mixture was allowed to stand 12 hours in the cold and then at room temperature until most of the excess hydrogen bromide had evaporated.

The resulting solution was washed with water, dilute ferrous sulfate, sodium hydroxide, and water, dried, and distilled through a 3 ft. jacketed concentric tube column. There were obtained about 6 cc. of a fore run boiling between  $107-112^{\circ}$  at 65 mm.,  $n_D^{24}$  1.4562 to 1.4539, which gave a positive test for secondary bromide with alcoholic silver nitrate (15). The main fraction, b.p.  $112^{\circ}$  at 65 mm.,  $n_D^{24}$  1.4532, weighed 23 gm. (67 per cent of theory) and contained no detectable secondary bromide.

$C_{11}H_{17}Br$ (192).	Calculated.	C 49.77, H 8.81, Br 41.42
	Found	" 49.56, " 8.91, " 41.47

*Optical Rotation*.—In homogeneous substance in a 1 dm. tube,  $\alpha_D^{24} = +8.05^{\circ}$ ,  $d_4^{24}$  1.110,  $[\alpha]_D^{24} = +7.25^{\circ}$ ,  $[M]_D = +13.92^{\circ}$ .

Although this substance has not previously been prepared in optically pure form, its theoretical maximum molecular rotation has been calculated by Levene and Marker to be  $[M]_D +14.92^{\circ}$  (16). Assuming this value to be correct, although it may be high, our primary bromide, after correcting for 3 per cent inactive isomer, is calculated to contain less than 4 per cent of the *l*-antipode. This slight racemization presumably occurred during the coupling of the active Grignard reagent with allyl bromide, since Whitmore and Olewine have shown that about 10 per cent racemization occurs in the cycle: active alcohol  $\rightarrow$  active chloride  $\rightarrow$  Grignard reagent  $\rightarrow$  active alcohol (17).

*8-Carbomethoxyoctanal-1*.—This compound was prepared in 40 per cent yield by ozonization of methyl oleate as previously described (18), b.p.  $105^{\circ}$  at 2 mm.,  $n_D^{25}$  1.4378.

*Methyl-d-14-methyl-9-hydroxypalmitate*. The Grignard reagent, prepared from 11 gm. of *d*-1-bromo-5-methylheptane and 3 gm. of magnesium in 80 cc. of ether, was added through a dropping-funnel to a stirred solution of 10.5 gm. of 8-carbomethoxyoctanal-1 in ether under dry nitrogen at  $0^{\circ}$ . The aldehyde ester had been extracted with dilute potassium hydroxide and freshly redistilled before use. Stirring was continued until hindered by the deposit of a tenacious white gummy precipitate. The mixture was allowed to stand 2 hours, and then decomposed with ammonium chloride solution, washed, dried, and distilled through a small Widmer column. Since the hydroxy ester was a mixture of diastereoisomers, it was not

further purified, b.p. 173–175° at 1.5 mm.,  $d_4^{25}$  0.920,  $n_D^{25}$  1.4512, yield 5.2 gm.

$C_{15}H_{30}O_2$  (300). Calculated, C 72.0, H 12.0; found, C 71.6, H 11.9

*Methyl-d-14-methylpalmitate*—To 5.2 gm. of the above hydroxy ester was gradually added 0.85 cc. of redistilled phosphorus tribromide at 0°. After standing for  $\frac{1}{2}$  hour the mixture was heated on the steam bath for  $\frac{1}{2}$  hour, decomposed with cold water, and extracted with ether. The washed ether solution was evaporated and the residue, which was not distillable under ordinary conditions, was allowed to stand for 12 hours with 9 gm. of potassium hydroxide in 45 cc. of 95 per cent alcohol, and then refluxed for 3 hours. The solution was then concentrated *in vacuo*, diluted with water, acidified, and extracted with petroleum ether. The crude unsaturated acids that were obtained from the petroleum ether

TABLE II  
*Distillation of Crude Methyl-d-14-methylpalmitate, Pressure 1.8 Mm.*

Fraction No.	Pot temperature	Jacket temperature	Still head temperature	B.p.	Volume	Refractive index, $n_D^{25}$
	°C.	°C.	°C.	°C.	cc.	
1	145	102	108	108	0.2	
2	160	136	140	146	0.1	
3	165	140	142	156	0.1	1.4339
4	164	140	143	156	0.3	1.4330
5	165	141	144	156	0.9	1.4334
6	169	145	144	156–160	0.4	1.4340
7	170–210	150		168	0.2	1.4353

extract by evaporation were dissolved in 15 cc. of dry methanol and hydrogenated at 30 pounds pressure with platinum oxide catalyst. Without isolating the reduced acids, the filtered solution was diluted with an additional 85 cc. of dry methanol containing 3 cc. of concentrated sulfuric acid and refluxed for 3 hours. From this solution there was obtained, after concentration *in vacuo* and extraction of the diluted residue with petroleum ether, 3.7 gm. of crude saturated methyl ester. This material was carefully distilled through a small fractionating column of a type previously described (19) but equipped with a vacuum jacket and thermocouples. The distillation is summarized in Table II. The boiling point of the main fraction was 156° at 1.8 mm. Weitkamp reported 156° at 1.9 mm. for the natural product.

*Optical Rotation*—In homogeneous substance in a 1 dm. tube,  $\alpha_D^{27} = +3.90^\circ$ ,  $d_4^{27}$  0.868,  $[\alpha]_D^{27} = +4.49^\circ$ .

$C_{15}H_{30}O_2$  (284). Calculated, C 76.05, H 12.68; found, C 75.85, H 12.57

*d*-14-Methylpalmitic Acid—0.9 gm. of the methyl ester was heated on the steam bath with 2 gm. of KOH in 8 cc. of 80 per cent alcohol for 1 hour. The alcohol was partially removed *in vacuo* and the residue diluted with water and extracted with ether to remove any neutral material. The solution was then acidified and extracted with petroleum ether. There was obtained from the washed and dried petroleum ether solution on evaporation 0.85 gm. of acid, which deposited flat needle crystals and then set to a solid crystalline mass. In order to remove traces of solvent the free acid was distilled in a small molecular still at 0.1 mm. pressure. The acid melted at 36.0–36.8°.

*Optical Rotation*—0.4248 gm. diluted to 3.0 cc. in acetone,  $\alpha_D^{20} = +0.73^\circ$  in a 1 dm. tube,  $[\alpha]_D^{20} = +5.16^\circ$ .

$C_{17}H_{34}O_2$  (270). Calculated, C 75.55, H 12.59; found, C 75.49, H 12.60

The rotation of a sample of the natural acids, provided by Dr. Weitkamp, was determined in the same manner, 0.49906 gm. in 3.0 cc. of acetone,  $\alpha_D^{20} = +0.87^\circ$ ,  $[\alpha]_D^{20} = +5.23^\circ$ . There was no significant depression in the mixed melting point of the synthetic and natural acid.

*Amide of d*-14-Methylpalmitic Acid—0.1 gm. of the acid was heated with 2 cc. of pure thionyl chloride on the steam bath for 1 hour in a dry, all glass system. The excess thionyl chloride was removed *in vacuo* and 10 cc. of concentrated ammonium hydroxide were added to the straw-colored residue and allowed to stand for 1 hour. The crude amide was filtered and recrystallized from 90 per cent methanol from which it was obtained in radiating clusters of needles, m.p. 88–89.5°. The amide prepared in the same manner from a sample of the natural acid melted sharply at 90.6°, mixed m.p. 89.2–89.4°. X-ray diffraction photographs were taken of specimens of the amides which had been melted between 1 cm. glass cover-slips and allowed to cool slowly from one end. Both specimens yielded the first, third, and fifth orders of an identical long spacing,  $32.8 \pm 0.3$  Å. The diffraction photograph from the amide of the synthetic acid was more diffuse than that of the natural product, and the intensities fell off more rapidly, indicating in agreement with the melting point behavior that the slight observed differences were due to traces of impurities, but that the substances were otherwise identical.

$C_{17}H_{35}ON$  (269). Calculated, N 5.21; found, N 5.26 (Kjeldahl)

It is a pleasure to acknowledge the suggestions of Dr. Philip G. Stevens.

#### SUMMARY

1. *d*-14-Methylpalmitic acid has been synthesized, starting from the *d*-2-methylbutanol-1 of fusel oil.

2. The synthetic acid is identical with the natural acid isolated from wool.

3. The proposed structure and configuration of the so called *antiseo* acids of wool fat are thus confirmed.

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# THE INFLUENCE OF INSULIN AND EPINEPHRINE UPON SOME PHOSPHORUS FRACTIONS OF THE BLOOD\*

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The relationships existing between the hormones regulating carbohydrate metabolism and the phosphorus compounds involved in carbohydrate metabolism are gradually becoming elucidated. We have studied the effect of the administration of insulin, epinephrine, and glucose to intact guinea pigs upon the inorganic, total acid-soluble, and barium-soluble alcohol-insoluble phosphorus of the blood, with labeled phosphate. In no case did the injection of any of these substances bring about a significant change in the specific activity of these phosphorus fractions.

## EXPERIMENTAL

Guinea pigs (385 to 550 gm.), which had been fasted 8 to 15 hours, received a single intraperitoneal injection of  $\text{Na}_2\text{HPO}_4^*$  (400,000 to 1,500,000 counts per minute on our scale-of-four Geiger-Müller counters) at intervals ranging from  $\frac{1}{2}$  to 48 hours before a blood sample was taken by cardiac puncture. Ether anesthesia was not satisfactory, since the animals struggled and there was a transitory rise in the blood sugar level from 123 mg. per cent to 130 to 155 mg. per cent. Urethane with dial, injected 30 minutes before the puncture, induced deep anesthesia without a change in blood sugar; dial anesthesia of longer duration (2 to 4 hours) caused a drop in blood sugar from 123 to 103 mg. per cent. The specific activity of the barium-soluble phosphorus, precipitated with alcohol, was the same in the ether and dial series.

In thirty-two animals, 0.5 to 1.0 I.U. of insulin per 100 gm. of body weight injected 90 minutes before the puncture caused an average decrease in the blood sugar level from  $123 \pm 0.8$  to  $52 \pm 16.5$  mg. per cent. The epinephrine-treated animals received 0.03 mg. of Parke, Davis adrenalin chloride per 100 gm. of body weight 60 to 90 minutes before the blood was sampled. In a series of twenty-one animals the blood sugar rose to an average value of  $167 \pm 14.9$  mg. per cent. Only the data from animals which responded to hormone treatment by a significant change in blood sugar have been included in the results.

\* This work was supported by a grant from the John and Mary R. Markle Foundation.

Aliquots of oxalated blood were used for the determination of the blood sugar by the Somogyi modification of the method of Shaffer and Somogyi (1). The inorganic phosphate was isolated as the strychnine phosphomolybdate from a 5 per cent trichloroacetic acid extract of 1 to 2 cc. of blood; the radioactivity was measured and the phosphorus content determined by the method of Tisdall adapted to the photoelectric colorimeter (2). The strychnine method was preferable to the precipitation of  $\text{Mg-NH}_4\text{PO}_4$  because of the small amount of blood available. The strychnine precipitate probably contained small amounts of phosphorus derived from the hydrolysis of the labile phosphate esters. The procedure was carried out in the cold as rapidly as possible.

Another aliquot of the 5 per cent trichloroacetic acid extract was used for the measurement of the radioactivity and phosphorus content of the total acid-soluble phosphorus (3). The results for inorganic and total acid-soluble phosphorus have been expressed as specific activity, the per cent of the original dose of  $\text{P}^*$  per mg. of phosphorus (4).

The procedure of Cori and Cori (5) for the isolation of hexose monophosphate from muscle was used for the precipitation of the barium-soluble, alcohol-soluble phosphorus from blood. This fraction will be referred to as the alcohol precipitate, A. P. According to Cori's and our own experiments, the final alcohol precipitate is contaminated with small amounts of inorganic phosphorus, which in our own case were highly radioactive. Therefore, non-radioactive phosphate was twice added to the barium-soluble extract and removed with  $\text{Ba}(\text{OH})_2$  before the addition of alcohol. In control experiments, non-radioactive hexose monophosphate was added to artificial trichloroacetic acid extracts of blood which contained inorganic phosphate in the approximate concentration and radioactivity of our blood extracts. Without dilution of the inorganic phosphate, 2 to 3 per cent of the inorganic phosphate accompanied the hexose monophosphate isolated from these extracts; in a group of our short term experiments, this contamination would have accounted for roughly 15 to 50 per cent of the  $\text{P}^*$  of the hexose monophosphate fraction. After dilution of the inorganic phosphate, the alcohol precipitate contained no  $\text{P}^*$ . The radioactive alcohol precipitate isolated from guinea pig blood was added to the trichloroacetic acid extract of blood from animals which had not received  $\text{P}^*$ ; no  $\text{P}^*$  was detectable in the inorganic phosphate isolated from these preparations. This indicates that we are probably dealing with contamination rather than an exchange occurring during the course of the isolation. The recovery of hexose monophosphate, however, was considerably reduced by the dilution procedure.

The extract containing the barium-soluble phosphorus was concentrated *in vacuo* to 5 cc. before addition of the alcohol. A ratio of water solution

to 95 per cent alcohol of 1:5 to 1:6 was found to be satisfactory. As it is not possible to isolate and analyze the A. P. in 1 day, the stability of hexose monophosphate and the A. P. of guinea pig blood was determined under a number of conditions. As judged by the sugar and phosphorus content, the barium-soluble extract can be kept in the ice box for 5 days without an appreciable change. Recovery was not affected by the length of time allowed for the alcohol precipitation (1 to 24 hours). The hydrochloric acid solution of the A. P. was stable in the cold for a week; some loss occurred upon reprecipitation with alcohol.

The A. P. was dissolved in  $\text{HCl}$  and its radioactivity measured. Removal of the barium had no effect upon the radioactivity or sugar values. Reducing sugar was determined in duplicate (1), also inorganic and total phosphorus whenever there was sufficient material (3). Since the barium, phosphorus, and sugar content of the alcohol precipitate corresponded closely to that of barium hexose monophosphate, the alcohol precipitate was probably largely made up of hexose monophosphate. The values for radioactivity are expressed as specific activity (in this case, the per cent of the original dose of  $\text{P}^*$  per gm. of reducing sugar of the alcohol precipitate).

### Results

The incorporation of  $\text{P}^*$  into the A. P. of the blood was investigated at various times after the administration of labeled phosphate.  $\text{P}^*$  appears rapidly in the blood A. P.; at 30 minutes, the earliest time investigated, the specific activity was considerable. The curve rises rapidly for the first 2 hours, reaches a maximum around 6 hours, and falls off gradually for the duration of the experiment, 48 hours. Since there is little difference among the 4, 6, and 12 hour average values (21.4, 26.0, and 25.3, respectively), there is no well defined maximum.

*Effect of Insulin, Epinephrine, and Glucose Administration.*—Injection of the hormones caused a reduction in the inorganic phosphorus content of the blood from a control level of  $5.5 \pm 0.4$  to  $4.0 \pm 0.8$  and  $4.0 \pm 0.3$  mg. per cent of P for the insulin and epinephrine series, respectively. Despite this decrease in amount, neither hormone caused a change in the specific activity of the inorganic phosphorus at any time after  $\text{P}^*$  injection (Fig. 1). This is in accord with Kaplan and Greenberg's finding for insulin 90 minutes after insulin and  $\text{P}^*$  administration (6).

Unlike Greenberg, we were not able to demonstrate a reduction in the specific activity of the total acid-soluble phosphorus after insulin treatment (Fig. 2). The turnover curves for the insulin and control groups were practically identical. The decrease in the total acid-soluble phosphorus content of the blood after insulin administration from  $30.2 \pm 1.0$  to  $27.8$



mg. per cent of P is not significant; a considerable part of the reduction can be accounted for by the decrease in inorganic phosphorus.

Epinephrine did not influence the amount of A. P. isolated from the blood in a group of thirty-four guinea pigs; the mean value for the control animals was  $5.0 \pm 0.5$ , and that of the epinephrine-treated animals was  $5.0 \pm 0.9$  mg. of reducing sugar isolated as A. P. per 100 cc. of blood. The corresponding value for the insulin-treated group (twenty-three animals) was somewhat greater ( $6.1 \pm 0.5$  mg. of sugar per 100 cc. of blood). Since the recovery of barium hexose monophosphate was considerably reduced in these experiments by the dilution procedure, these results

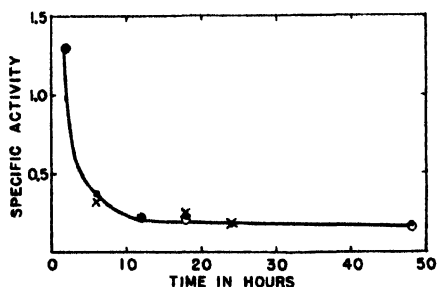


FIG. 1

FIG. 1. Mean specific activity of control animals (●), insulin-treated animals (○), and epinephrine-treated animals (X). The specific activity is the per cent of the dose of  $P^*$  per mg. of inorganic phosphorus isolated from the blood at various times after  $P^*$  injection.

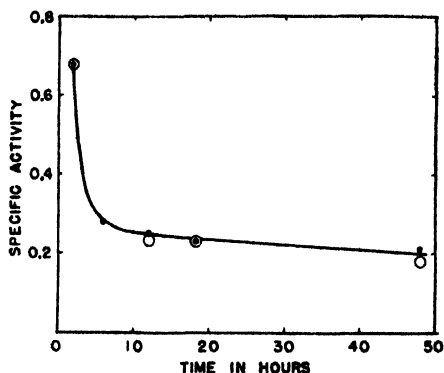


FIG. 2

FIG. 2. Mean specific activity of control animals (●), and insulin treated animals (○). The specific activity is the per cent of the dose of  $P^*$  per mg. of total acid-soluble phosphorus of the blood measured at various times after  $P^*$  injection.

would serve only to indicate a trend. Therefore, despite the statistical insignificance of the difference in values, insulin probably causes an increase in the A. P. content of the blood.

The effect of insulin on the specific activity of the blood A. P. was studied at a number of times after  $P^*$  injection in order that we might compare the turnover curves of the control and insulin-treated groups. The results are summarized in Table I. At 1, 2, and 3 hours, when the uptake of  $P^*$  by the A. P. is rapid, there is no difference in the specific activity of this fraction of the blood in the control and treated animals. This confirms the work of Kaplan and Greenberg who showed that insulin did not influence the specific activity of the barium-soluble phosphorus of the blood

of the rabbit at 90 minutes after injection of P\* and insulin (6). Nor is there a significant difference at 12 hours, when the control curve was close to its maximum, or at 18 hours, when the curve is falling off.

Likewise, there was no difference between the specific activity of the A. P. of the epinephrine-treated and control animals at 3, 6, 18, and 24

TABLE I  
*Specific Activity\* of Alcohol Precipitate of Blood*

Time <i>hrs.</i>	Controls		Insulin treated		Adrenalin-treated	
	No. of animals	Specific activity	No. of animals	Specific activity	No. of animals	Specific activity
$\frac{1}{2}$	2	$8.4 \pm 0.8$				
1	4	$12.4 \pm 1.2$	4	$14.1 \pm 1.1$		
2	8	$19.4 \pm 4.4$	10	$20.1 \pm 1.9$		
3	4	$18.1 \pm 2.3$	2	$19.0 \pm 1.9$	4	$16.6 \pm 1.2$
4	2	$24.4 \pm 1.6$				
6	5	$26.0 \pm 3.0$			4	$25.0 \pm 5.2$
12	3	$25.3 \pm 1.9$	2	$23.4 \pm 1.6$		
18	9	$22.5 \pm 1.1$	4	$20.9 \pm 3.2$	4	$21.9 \pm 2.2$
24	4	$21.8 \pm 1.8$			4	$20.5 \pm 1.2$

\* Per cent of the dose of P\* per gm. of reducing sugar of the alcohol precipitate.

TABLE II  
*Time of Epinephrine Injection and Specific Activity\* of Alcohol Precipitate*

Time after epinephrine administration <i>hrs.</i>	No. of animals	Specific activity
0	4	$21.8 \pm 1.8$
$\frac{1}{2}$	1	23.4†
1	4	$20.5 \pm 1.2†$
2	2	$21.7 \pm 0.1†$
3	2	$23.5 \pm 0.1†$
4	2	$21.5 \pm 0.9$

\* Per cent of the dose of P\* per gm. of reducing sugar of the alcohol precipitate.

† The average includes values which were corrected for a discrepancy in the P\* decay curve.

hours after P\* injection. Cori and Cori (7) found that epinephrine caused an accumulation of hexose monophosphate in rat muscle; this increase was apparent 15 minutes after injection, maximal around 1 hour, and it had disappeared by 4 hours. It was possible, therefore, that, although there was no effect at the time selected for the administration of epineph-

rine (60 to 90 minutes prior to the puncture), a change might be detectable at other times. Animals, which received P\* 24 hours before the blood was sampled, were given epinephrine at times ranging from  $\frac{1}{2}$  to 4 hours before the puncture (Table II). At none of these times was there a difference in the specific activity of the blood A. P. attributable to the hormone.

The effect of glucose, given orally or parenterally, alone or in conjunction with insulin on the specific activity of the A. P. was investigated in a group of animals which received P\* 2 hours before the blood sample was taken. The values for the glucose-treated animals (17.8 to 22.2) fell within the range of values for the corresponding control and insulin-treated groups.

None of the procedures employed, administration of insulin, epinephrine, glucose alone or in conjunction with insulin, influenced the specific activity of the A. P. of the blood.

#### DISCUSSION

The investigation of the influence of insulin and epinephrine on the phosphorus compounds of the blood has been complicated by the similar results obtained with the two antagonistic hormones. In the intact animal, both hormones cause a reduction in the amount of inorganic phosphorus in the blood and an accumulation of hexose monophosphate in the muscle. The decrease in blood inorganic phosphorus is attributable to insulin, since it does not occur in the depancreatized dog treated with epinephrine (8). The accumulation of hexose monophosphate in muscle is attributable to epinephrine, since it does not occur in the adrenalectomized animal treated with insulin (7). In the normal animal it is difficult to differentiate between insulin and epinephrine action in these respects, as hyperglycemia appears to cause a stimulation of the pancreas and hypoglycemia a stimulation of the adrenals.

In our experiments both hormones cause a reduction in the blood inorganic phosphorus (the decrease was of the same magnitude, 1.5 mg. per cent of P for both insulin and epinephrine) without a change in specific activity.

Neither insulin, epinephrine, nor glucose influenced the specific activity of the blood hexose monophosphate. Kaplan and Greenberg (6) have shown that insulin results in an accumulation of hexose monophosphate in the blood; our results indicate a trend in the same direction with insulin, but not with epinephrine. In contrast to the blood picture, both hormones produce a rise in the amount of hexose monophosphate in the muscle and insulin causes an increase in the specific activity of the muscle hexose monophosphate (6, 7).

The author wishes to express her appreciation to Dr. John R. Murlin and Dr. William Bale for their helpful advice and interest in this work.

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#### SUMMARY

1. The influence of insulin, epinephrine, and glucose administration upon several phosphorus fractions of the blood of intact guinea pigs has been investigated with labeled phosphate.

2. The turnover curve for the barium-soluble, alcohol-insoluble phosphorus was established. None of the procedures employed influenced the specific activity of this fraction.

3. Both insulin and epinephrine caused a decrease in the amount of inorganic phosphorus without a change in specific activity.

4. Neither the amount nor the specific activity of the total acid-soluble phosphorus was affected by insulin injection.

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# THE MECHANISM OF ACTION OF THE ANTIFATTY LIVER FACTOR OF THE PANCREAS

## II. FREE METHIONINE PREVENTS FATTY LIVERS IN COMPLETELY DEPANCREATIZED DOGS MAINTAINED WITH INSULIN AND FED A LEAN MEAT DIET\*

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The results of an earlier investigation demonstrated that complete excision of the pancreas or, more specifically, loss of the external secretion of the pancreas profoundly affects choline metabolism (1). In the absence of the external secretion of the pancreas there occurs a reduction in the concentration of plasma or circulating choline. Depletion of choline stores in the body would, therefore, appear to provide a reasonable explanation for the development of fatty livers in such animals. This view is in keeping with the observation that it required the addition to the diet of at least 35 mg. of free choline per kilo of body weight per day to prevent the development of fatty livers in dogs deprived of their pancreas but kept alive with insulin (2).

In seeking to interpret these findings we are confronted at the outset with the fact that the development of the syndrome involving fatty liver and depletion of circulating choline in the dog deprived of the external secretion of the pancreas is not dependent upon a lack of methyl donors in the diet. This syndrome appeared in dogs that were fed 500 gm. of lean meat per day; this contains about 0.5 gm. of choline and 3 gm. of methionine, an amount of lipotropic substances that is more than sufficient for prevention of fatty livers in normal dogs. *Interestingly enough, this amount of lean meat is also sufficient to prevent the development of fatty livers in completely depancreatized dogs, provided they ingest once daily 60 mg. of Fraction 27C derived from raw pancreas* (3).

The above considerations suggest that in the dog deprived of the external secretion of the pancreas there is an interference either in the mechanism by which bound choline and methionine of the diet are made available for lipotropic purposes or in the synthesis of choline from methyl donors. Either interference could account for the decreased amounts of choline found in plasma.

The present investigation bears on the question of synthesis of choline by the depancreatized dog maintained with insulin.

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## EXPERIMENTAL

The five dogs used in this study were depancreatized and then fed twice daily a lean meat-sucrose diet supplemented with 50 gm. of pancreas per meal, as outlined in a previous paper (4). 1 week after pancreatectomy all dogs had recovered from the operation and had excellent appetites. The feeding of raw pancreas to all five dogs was then discontinued. During the next 20 weeks Dogs D515, D516, and D517 received respectively 1.05, 1.3, and 1.5 gm. of *dl*-methionine with each meal. Dogs D526 and D532 served as controls and hence received no supplements in their diets. At the end of this time the dogs were sacrificed, and their livers excised and analyzed for lipids in a manner already described (5).

TABLE I  
*Liver Lipids of Depancreatized Dogs Fed dl-Methionine for 20 Weeks*

	Dog No.	Body weight			Period of observation (21 weeks) after pancreatectomy			Liver				
		Pre-operative	At start of methionine feeding	Final	Fed pancreas	Methionine-fed		Weight	Total fatty acids	Phospholipids		
						Period	Amount			Total	Choline-containing	Non-choline-containing
		kg.	kg.	kg.	wks.	wks.	gm. per day	gm.	per cent	per cent	per cent	per cent
Fed methionine	D515	7.9	7.5	5.5	1	20	2.1	238	3.0	1.95	0.86	1.09
	D516	9.7	9.7	9.1	1	20	2.6	412	6.7	1.67	0.70	0.97
	D517	6.9	7.0	7.2	1	20	3.0	358	2.8	1.90	1.21	0.69
Control	D532	10.5		7.6	1			312	22.0	1.52	0.72	0.80
	D526	8.6		6.0	1			376	17.7	1.47	0.63	0.84

*Results*

It was shown earlier that approximately 35 mg. per kilo of body weight per day comprised the minimum amount of choline required to prevent the infiltration of abnormal amounts of fat in the liver of the depancreatized dog fed a meat-sucrose diet (2). The amount of methionine added to the diets of the three depancreatized dogs (Table I) was based on this choline value. Dogs D515 and D516 were fed 260 mg. of methionine per day per kilo of initial body weight; this amount contained enough methyl for the synthesis of about twice the minimum effective dose of choline. The amount of methyl in the methionine-fed dog, No. D517, namely 430 mg. per kilo per day, was sufficient for the synthesis of approximately 3 times the minimum effective dose of choline.

The results recorded in Table I show that the enteral administration of methionine prevents the development of fatty livers in depancreatized dogs fed a lean meat-sucrose diet. The daily feedings of methionine were continued for 20 weeks, since it was found that a test period of 16 to 20 weeks was necessary to determine whether a substance possessed antifatty liver activity in the depancreatized dog (4).

It is of interest to note that two of the three dogs lost little or no weight during the 20 week period. The third dog (No. D515), however, lost about 30 per cent of its preoperative weight during the 5 months that it received methionine.

In Dogs D515 and D516 the concentration of choline-containing phospholipids of the liver was somewhat lower than that usually found in normal livers. No significance, however, can be attached to this finding at the present time.

#### DISCUSSION

According to du Vigneaud *et al.*, methionine prevents fatty infiltration in the liver by virtue of its labile methyl groups which the body uses for the synthesis of choline (6-8). These workers have furnished indisputable proof of the transfer of methyl groups from methionine to choline in the rat, rabbit, and man. The recent finding of McKibbin *et al.* that methionine is lipotropic in weanling pups (9) leaves no doubt that methionine is also a precursor of the methyl groups of choline in the dog.

It is shown in the present investigation that the administration of methionine prevents the development of fatty livers in depancreatized dogs maintained with insulin and fed a lean meat diet. In view of the metabolic interrelations of choline and methionine discussed above, it is reasonable to conclude that the synthesis of choline from methionine also proceeds in the absence of the external secretion of the pancreas.

It has been shown that pancreas fractions that contain the antifatty liver factor have a profound effect on choline metabolism. The fall in circulating choline that occurs in dogs deprived of the pancreas can be prevented by the addition to their diets of as little as 60 mg. of Fraction 27C (1). While the mechanism of this action is not clear at present, the results of this investigation, along with the earlier finding that ingested choline prevents fatty livers in depancreatized and duct-ligated dogs, serve to rule out the possibility that the antifatty liver factor of the pancreas is concerned with either of two phases of choline metabolism: its synthesis in the animal body and its action upon the liver as a lipotropic agent.

Since the synthesis of choline from *free* methionine is not interfered with in the depancreatized dog maintained with insulin, the most plausible explanation of the action of the antifatty liver factor of the pancreas would



appear to be that it makes available for lipotropic purposes the *bound* methionine contained in dietary protein. The results presented here are therefore not inconsistent with the concept that the antifatty liver factor is enzymatic in nature. As a working hypothesis it may be considered that this factor is a proteolytic enzyme without which ingested proteins cannot exert their lipotropic action.

#### SUMMARY

1. The development of fatty livers in the depancreatized dog maintained with insulin and fed a lean meat diet can be prevented by the addition of *free* methionine to the diet.

2. An explanation of the action of the antifatty liver factor of the pancreas is offered.

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# STUDIES ON VITAMIN B<sub>6</sub> CONJUGASE FROM CHICKEN PANCREAS\*

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Recently Laskowski, Mims, and Day (1) described a method leading to the partial purification of the enzyme which produces the *Streptococcus faecalis* (*S. lactis* R) growth-stimulating factor from inactive precursor substance in yeast. At about the same time Bird *et al.* (2) described an enzyme obtained from hog kidney which appears to catalyze the same reaction, and suggested vitamin B<sub>6</sub> conjugase as a provisional name for their enzyme.

In order to avoid confusion by using several names for insufficiently defined enzymes, all of which are evaluated by the same or similar methods, it was decided to use in this paper the provisional name vitamin B<sub>6</sub> conjugase and to specify the enzyme further by its origin. Therefore we refer to the enzyme described by Laskowski, Mims, and Day (1) as chicken pancreas conjugase, to the enzyme described by Bird *et al.* (2) as the hog kidney conjugase, and so on. It is not intended to imply that all these enzymes are identical. Chicken pancreas conjugase (1) showed an optimal pH between 7 and 8, hog kidney conjugase (2) at pH 4.5. Lemon and Totter (3) studied the kinetics of rat liver conjugase, chicken pancreas conjugase, and potato conjugase, and found the values for  $K_m$  (Michaelis constant) to be 0.29, 3.0, and 38.0 respectively.

Once the chemical structure of vitamin B<sub>6</sub> conjugate is known, it will be much easier to decide whether conjugases so widely distributed in nature are identical or not. At the present state of our knowledge it may be that conjugases differ not only in respect to the conditions of activity but also in respect to the mechanism of the reaction itself.

It was previously found (1) that chicken pancreas conjugase was rather resistant to tryptic digestion. This finding suggested the revision of the previously described method of purification because it seemed logical to expect that autolysis of pancreas may increase the amount of enzyme extracted and decrease the amount of inactive protein. This expectation was confirmed (4).

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The object of this paper is to present the improved method of preparation of chicken pancreas conjugase and to describe some properties of the purified enzyme.

### Methods

The methods of determination were the same as those described in the previous paper (1), except that in most of the experiments the concentrate of vitamin B<sub>6</sub> conjugate, kindly supplied by Dr. J. J. Pfiffner of Parke, Davis and Company, was used as a substrate. The product of the enzymic reaction was tested microbiologically with *Streptococcus faecalis*, American Type Culture Collection No. 8043, and the results were compared with those obtained with pure vitamin B<sub>6</sub>, also kindly supplied by Dr. Pfiffner.

In the previous paper (1) the product of the reaction was expressed in micrograms of *Streptococcus lactis* factor equivalent to micrograms of folic acid of the potency 40,000. Under the conditions employed it was found that 1  $\gamma$  of pure vitamin B<sub>6</sub> corresponded roughly to 4  $\gamma$  of folic acid. Concentration of substrate (vitamin B<sub>6</sub> conjugate) was always expressed in micrograms of free vitamin B<sub>6</sub> obtainable from the amount of substrate used. This value represented the maximal value obtainable after the completion of the enzymic reaction, carried out under the most favorable conditions with a huge excess of crude conjugase.

In a few experiments designed for the investigation of other suspected products of the reaction, a substrate was prepared from Difco yeast extract as follows: 20 gm. of Difco yeast extract were dissolved in 20 cc. of water in a 250 cc. centrifuge tube. 150 cc. of aluminum picrate reagent (Buell (5)) were added and the mixture was left to stand overnight in the ice box. It was centrifuged, the precipitate was decomposed with morpholine, reprecipitated with acetone (according to the directions of Buell (5)), and dried. In this step the yield was about 90 per cent, and the purification on a dry weight basis was 10-fold, which would be higher if corrected for the weight of aluminum. The dry aluminum salt was dissolved in about 70 cc. of cold 0.2 N HCl, the pH was adjusted to 3.5 with NaOH, and the mixture was centrifuged. The precipitate was washed with acetone and dried. In the second step only a 2-fold purification was achieved and the yield was only about 60 per cent. The product thus obtained showed comparatively low reducing power when measured by the method of Hagedorn and Jensen (6) as modified by Robison and Morgan (7).

Protein was determined by the method of Robinson and Hogden (8), and the potency of enzyme was expressed in units (see below) per mg. of protein.

### EXPERIMENTAL

*Preparation of Enzyme*—The method of preparation which was finally adopted consisted of the following steps.

*Autolysis*—2 kilos of frozen chicken pancreas<sup>1</sup> were first ground in a meat grinder, and then in a mortar with sand. 2 volumes of 0.1 M phosphate buffer, pH 8, were added. The mixture was covered with toluene and left for 24 hours at 32° to autolyze; the pH of the mixture was frequently checked and readjusted to 8 by addition of NaOH. After 24 hours the mixture was centrifuged and the liquid collected. It contained about 70,000,000 new units (see below); the potency was about 4000 units per mg. of protein.

As a result of autolysis the amount of enzyme present in the crude extract was increased almost 1000 times. Apparently, however, autolysis with 2 volumes of buffer led only to about 80 per cent extraction, because when the small amount of autolyzed pancreas was treated with 100 volumes of buffer about 20 per cent more conjugase was found in the extract. Assuming the amount of protein in the fresh pancreas to be 20 per cent and the amount of conjugase 50,000,000 units per kilo, the potency of the fresh pancreas should have been 250. Autolysis resulted in only a 16-fold increase in potency. The actual increase in potency in this step must have been considerably higher, because both protein and polypeptides were measured as protein by the method used. This became apparent from the results obtained in the next step, in which a very large quantity of peptides was removed in the filtrate of 80 per cent saturated ammonium sulfate.

*Salting-Out with Ammonium Sulfate*—The enzyme solution was treated with 30.2 gm. of ammonium sulfate per 100 cc. to make 40 per cent saturation; the pH was kept around 7 by addition of N NaOH. The heavy precipitate was filtered off and discarded. To the filtrate 25.8 gm. of additional ammonium sulfate were added per 100 cc. to make 80 per cent saturation. A small brownish precipitate was filtered off in the cold and was placed, together with the filter paper, in a dialyzing bag. It was dialyzed overnight against a large volume of phosphate buffer at pH 8 in the cold. The dialyzed extract still contained about 75 per cent of the activity of the original pancreas and the potency was increased about 100 times.

*Precipitation with Alcohol*—The dialyzed enzyme, cooled in an ice bath, was treated with an equal volume of 95 per cent alcohol previously cooled to -10°. The mixture was left in the ice bath for about 1 hour and was centrifuged in the cold. The precipitate was mixed with about 20 cc. of 0.2 M buffer at pH 7.8 and the denatured protein was centrifuged off and discarded. The solution contained 40,000,000 units; the potency was 500,000 units per mg. of protein.

*Second Precipitation with Ammonium Sulfate*—The solution was treated with solid ammonium sulfate to make 40 per cent saturation, and a few drops of N NaOH were added to readjust to pH 7. The brownish precipi-

<sup>1</sup> Obtained from the Chester B. Franz Company, Mammoth Spring, Arkansas.

tate was centrifuged down and discarded. To the filtrate solid ammonium sulfate was added to make 80 per cent saturation. The precipitate was collected and was dissolved in a minimal amount of buffer (about 8 cc.). As a result of this treatment the potency was only slightly increased but most of the color was removed.

Attempts to crystallize the enzyme by adding ammonium sulfate were not successful. Irregular needles were occasionally observed, mixed with a considerable amount of amorphous material. Recrystallization was unsuccessful. Table I summarizes the results of purification.

*Properties of Chicken Pancreas Conjugase*—The enzyme obtained by the present method showed very high initial velocity when minute amounts of enzyme were tested with a considerable excess of substrate. It required, however, enormous amounts of enzyme to bring the reaction to completion. Several possible causes could have been responsible for this fact. A

TABLE I  
*Summary of Potency and Yield of Enzyme after Various Steps in Purification Procedure with Use of New Units*

Steps in procedure	Potency per mg. protein	Yield	
		From 2 kilos fresh tissue	Per cent recovery
	<i>new units</i>	<i>new units</i>	
Crude tissue.....	250		
After autolysis.....	4,000	70,000,000	87
After 40-80% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> pptn.....	100,000	60,000,000	75
After alcohol pptn.....	500,000	40,000,000	50
After 2nd 40-80% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	700,000	20,000,000	25

likely one appeared to be the absence of some of the components of the enzymic system in the purified preparation. During the previous work on purification of the enzyme it was noticed that after the treatment with calcium phosphate gel the total amount of units recovered was almost invariably higher than prior to the calcium treatment. It was thought that calcium might be one of the missing components of the system. This hypothesis was confirmed. The results are shown in Table II. The optimal concentration of calcium was found to be 0.01 M, almost the limit of solubility in borate buffer. The effects of the addition of calcium on the time-activity curve are shown in Fig. 1. Addition of calcium not only increased the initial velocity of the reaction, but also shifted the apparent equilibrium considerably to the right. However, the amount of enzyme employed in this experiment (4 units) was not sufficient to bring the reaction to completion, even after the addition of calcium.

In the previous work (1) it was reported that dialysis against water resulted in a considerable loss of activity. This experiment was repeated and confirmed. However, when the dialyzed enzyme was tested in the presence of calcium the original activity was restored.

TABLE II  
*Effect of Calcium on Conjugase*

Each tube contained 1  $\gamma$  of vitamin B<sub>6</sub> conjugate in 1 cc. of 0.2 M borate buffer, pH 7.8, and 1:100,000 dilution of enzyme, plus the different calcium chloride concentrations. Incubated 1 hour at 32°.

Added calcium chloride in reacting mixture	Vitamin B <sub>6</sub> produced
M	$\gamma$
No calcium added	0.05
0.0001	0.05
0.001	0.25
0.01	0.50

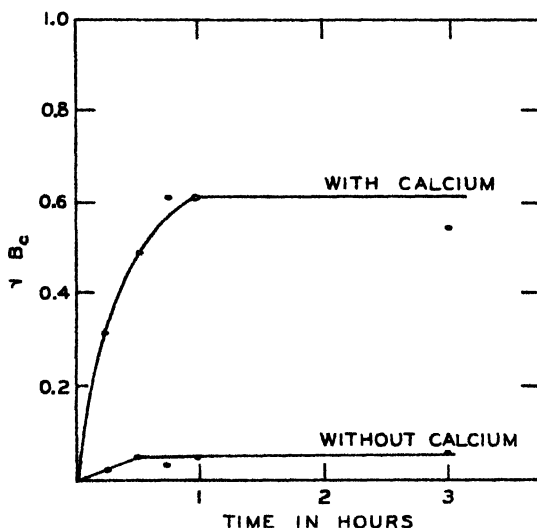


FIG. 1. Effect of calcium on the time-activity curve. Each tube contained 4 units of conjugase, 1  $\gamma$  of vitamin B<sub>6</sub> conjugate in 0.2 M borate buffer, total volume 1 cc. Half of the tubes contained 0.01 M calcium chloride. Temperature of incubation 32°.

Fig. 1 shows also that the time of incubation previously chosen for the determination of activity should be changed, because under the conditions described the reaction is completed within 1 hour. Other optimal conditions of activity were reinvestigated. Table III shows the influence of temperature and Fig. 2 the influence of pH on the reaction.

A new unit is therefore proposed. It is defined as 10 times the amount of enzyme which produces 0.1  $\gamma$  of vitamin B<sub>6</sub> when incubated with 1  $\gamma$

TABLE III

*Summary of Experiments upon Effect of Temperature on Conjugase Activity*

Each tube contained 1  $\gamma$  of vitamin B<sub>6</sub> conjugate in 1 cc. of 0.2 M borate buffer, pH 7.8, and 0.01 M CaCl<sub>2</sub> plus the enzyme. In each series the activity at 32° was taken as 100 per cent.

Temperature	Activity
°C.	<i>per cent</i>
18	12
24	40
32	100
37	75

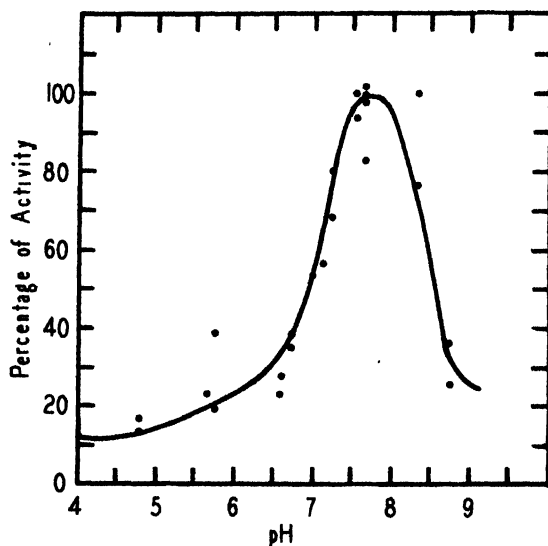


FIG. 2. Effect of pH on conjugase. Each tube contained 4 to 7 units of conjugase, 1  $\gamma$  of vitamin B<sub>6</sub> conjugate, and 0.01 M CaCl<sub>2</sub> in buffer solution (acetate, phosphate, borate of appropriate pH), total volume 1 cc. Incubation 1 hour at 32°. The results were obtained in several series of experiments. The highest value in each series was considered 100, and the other values of the same series were expressed in per cent thereof.

of vitamin B<sub>6</sub> conjugate during 1 hour at 32° in the presence of 0.01 M CaCl<sub>2</sub> in 0.2 M borate buffer, pH 7.8, total volume 1 cc.

In order to determine the amounts of units in 1 cc. of the enzyme prepara-

tion it was necessary to use the technique of serial dilutions. An example is shown in Table IV. Units per cc. of the original enzyme solution were calculated from the greatest dilution in which not less than 0.1  $\gamma$  and not more than 0.3  $\gamma$  of vitamin B<sub>6</sub> were produced.

From the results of Table IV and Fig. 1 it is obvious that, at the concentration of substrate of 1  $\gamma$  per cc., in spite of the addition of calcium the apparent equilibrium of the reaction lies close to 50 per cent of the substrate utilized. 20-fold amounts of enzyme of the second stage (after the first precipitation with  $(\text{NH}_4)_2\text{SO}_4$ ) and 400-fold amounts of enzyme of the fourth stage (after the second fractionation with  $(\text{NH}_4)_2\text{SO}_4$ ) failed to bring the reaction to completion.

TABLE IV  
*Determination of New Units*

Each tube contained 1  $\gamma$  of vitamin B<sub>6</sub> conjugate in 1 cc. of 0.2 M borate buffer, pH 7.8, and 0.01 M calcium chloride plus the different dilutions of enzyme. Incubated 1 hour at 32°.

Dilution of enzyme during incubation	Vitamin B <sub>6</sub> produced			
	After autolysis	First $(\text{NH}_4)_2\text{SO}_4$ ppt.	After alcohol pptn.	* Second $(\text{NH}_4)_2\text{SO}_4$ ppt.
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
Control, boiled, 1:50,000	0	0	0	0
Experimental, 1:50,000	0.6	0.5	0.5	0.5
1:200,000	0.1	0.5	0.5	0.6
1:1,000,000	0	0.5	0.5	0.5
1:5,000,000	0	0.3	0.4	0.5
1:20,000,000	0	0	0.2	0.3
1:100,000,000	0	0	0	0
	units per $\mu$ .	units per cc.	units per cc.	units per cc.
	20,000	1,500,000	4,000,000	6,000,000

One of the possible explanations of this finding may be the absence of another, not yet identified, component of the enzymic system. Several suspected substances were investigated but were found inactive. They were glutathione, cysteine, boiled blood plasma, digest of yeast nucleic acid by ribonuclease, digest of thymonucleic acid by thymonucleodepolymerase, and liver extract.

In a preliminary note (4) it was reported that purified preparations of conjugase were yellow, showing maximum absorption around 360  $m\mu$ . A series of ten preparations was compared for the intensity of absorption at 360  $m\mu$  and for conjugase activity. No correlation could be established. It seems therefore likely that the enzyme is colorless and that the yellow component represented an impurity.



*Attempts to Identify Enzyme*—Several attempts to identify the enzyme have been made. Bird *et al.* (2) reported that hog kidney conjugase was not identical with kidney nucleosidase, acid phosphatase of almond or potato, alkaline phosphatase of small intestine, or  $\beta$ -glucosidase of almond.

Through the courtesy of Dr. G. Schmidt we were able to test his highly purified phosphatase (9) and found no conjugase activity in it, which confirms the findings of Bird *et al.* (2).

The purified preparations of chicken pancreas conjugase exhibited some lipolytic activity. However, when several subsequent stages of purification were compared for activities of conjugase and of lipase (according to the method of Willstätter *et al.* (10)), the results were far from being parallel (Table V). Attempts to show the liberation of the acidic groups from vitamin B<sub>6</sub> conjugate were also unsuccessful. The negative results of these experiments should not be interpreted as a definite proof that conjugase

TABLE V  
*Comparison of Conjugase and Lipase Activity of Several Enzyme Preparations*

Enzyme preparation		Conjugase	Titration of fatty acids	Ratio expected	Ratio found
cc.		units	cc. 0.2 N NaOH		
1	B	20,000	3.0	1:1:1	1:0:0
1	D (1:375)	20,000	0.0		
1	E (1:2000)	20,000	0.0		
2	A	4,000	1.4	0.2:1:10	0.5:1:0.15
1	B	20,000	2.7		
10	D (1:37.5)	200,000	0.4		

is not an esterase. It may be that different optimal conditions are required for activity on different substrates. It is also possible that minute quantities of acid were not detected.

More encouraging results were obtained by comparing the conjugase activity of our enzyme with its activity in releasing reducing substances. In the first series of experiments the digest of purified commercial yeast nucleic acid with ribonuclease (prepared according to Kunitz (11)) was used as a substrate. A small increase of the reducing power was found invariably when such a digest was incubated with conjugase. The increased reducing power, however, accounted for not more than 1 per cent of the value to be expected, if all the ribose present were liberated. When several subsequent stages of purification of conjugase were tested for nucleosidase activity on this substrate, and compared with the conjugase activity determined on B<sub>6</sub> conjugate, both activities increased with the increasing purification, but no quantitative relationship could be established.

In another series of experiments the previously described nucleotide fraction from Difco yeast extract was used as a substrate. Several preliminary experiments were made in which the experimental tube contained substrate, enzyme, calcium chloride, and buffer. The control tube contained the same components except that the enzyme was previously boiled for 5 minutes. Both tubes were incubated for the same length of time, and were analyzed for both vitamin B<sub>6</sub> and a reducing substance. In all cases the reducing substance and vitamin B<sub>6</sub> were higher in the experimental

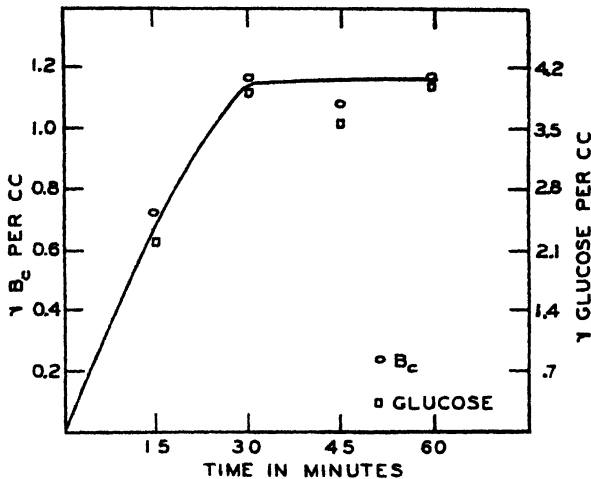


FIG. 3. Comparison of rate of release of a reducing substance and of vitamin B<sub>6</sub>. The reaction mixture contained 400 mg. of "nucleotide fraction obtained from Difco yeast extract" as a substrate, 1,000,000 new units of conjugase in 0.2 M borate buffer, pH 7.8, made 0.01 M in respect to CaCl<sub>2</sub>, total volume 60 cc. 10 cc. samples were withdrawn at 15 minute intervals. The reducing power is expressed in micrograms of glucose per cc., and the growth-stimulating substance in micrograms of vitamin B<sub>6</sub> per cc.

tubes; however, the ratio between the two varied on different days from 3 to 7 γ of glucose per 1 γ of vitamin B<sub>6</sub>.

Fig. 3 shows an experiment in which the time-activity curve was run simultaneously for both reducing substance and vitamin B<sub>6</sub>. The result of this experiment indicated that both were liberated at the same rate.

The reducing substance liberated in the course of this experiment could have been derived from substances other than vitamin B<sub>6</sub> conjugate. However, when one considers that the substrate was obtained by precipitation of nucleotides similar to adenylic acid (specificity of Buell reagent), it seems highly probable that both substances were liberated from compounds of similar structure, if not from the same. It is of course possible

that the reducing substance is not a sugar at all. This question could not be answered on the basis of our experiments, because neither isolation nor identification could be attempted with the amounts of substrate available.

Experiments similar to those previously described, in which both activities were compared at different time intervals, were made with vitamin B<sub>6</sub> conjugate as a substrate. The results are shown in Fig. 4. This experiment differed from the previous time-activity experiments in that equilibrium was not reached within 1 hour. This could have been due to the large volume of liquid used in this experiment (120 cc. instead of 1 cc.) which slowed down the adjustment to temperature in the air incubator.

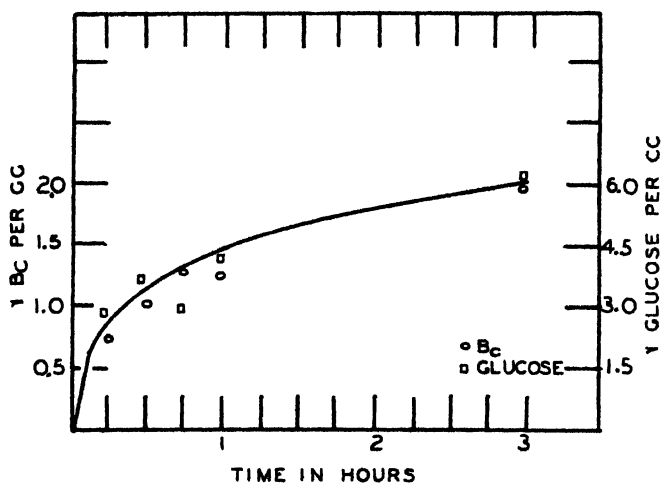


FIG. 4. Comparison of the rate of release of a reducing substance and of vitamin B<sub>6</sub>. The reaction mixture contained 480  $\gamma$  of vitamin B<sub>6</sub> conjugate as a substrate, 1,000,000 new units of conjugase in 0.2 M borate buffer, pH 7.8, made 0.01 M with respect to CaCl<sub>2</sub>, total volume 120 cc. 20 cc. samples were withdrawn at the intervals shown. The reducing power is expressed in micrograms of glucose per cc., and the growth-stimulating substance in micrograms of vitamin B<sub>6</sub> per cc.

In spite of the slower reaction the rate of liberation of reducing substance paralleled the liberation of vitamin B<sub>6</sub>.

One experiment was made on crystalline *Lactobacillus casei* factor, kindly sent to this department by Dr. Stokstad of the Lederle Laboratories, Inc. Only 0.5  $\gamma$  of reducing substance expressed as glucose was liberated in this experiment per 1  $\gamma$  of the growth-stimulating factor measured as vitamin B<sub>6</sub>.

#### SUMMARY

An improved method of preparation of chicken pancreas conjugase is described. The method consists of 24 hours autolysis at pH 8 at 32°,

fractional precipitation with ammonium sulfate between 40 and 80 per cent saturation at pH 7, precipitation with alcohol, and a second fractionation with ammonium sulfate.

The presence of calcium was found to activate chicken pancreas conjugase, the optimal concentration being 0.01 M. Optimal pH and temperature were found to be 7.8 and 32° respectively.

A new unit of conjugase is proposed as 10 times the amount of enzyme which produces 0.1  $\gamma$  of vitamin B<sub>6</sub> when incubated with 1  $\gamma$  of vitamin B<sub>6</sub> conjugate for 1 hour at 32° in the presence of 0.01 M CaCl<sub>2</sub> in 0.2 M borate buffer, pH 7.8, total volume 1 cc.

Increase of reducing power was observed as a result of incubation of the conjugase with several different substrates and was found to parallel the release of vitamin B<sub>6</sub>, but no decisive evidence was accumulated to show that this was due to a release of a sugar.

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# THE EFFECT OF DIETARY PROTEINS AND AMINO ACIDS ON LIVER FAT

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The recognition of protein as a dietary factor affecting the outcome of investigations involving lipotropic substances (1-5) and the subsequent discovery that cystine (6) and methionine (7) exert marked effects on the level of liver fat in rats on low choline diets occasioned several attempts to determine whether these two amino acids alone were involved or whether others were also concerned in the phenomenon. Best and Ridout (8) and Channon, Manifold, and Platt (9) reported that dietary casein exerts a distinctly stronger lipotropic effect than does the methionine and cystine contained in it (suggesting the presence in protein of other active constituents), but Tucker, Treadwell, and Eckstein (10) found just the opposite. 2 years later Treadwell, Groothuis, and Eckstein (11) published further experimental data indicating that the free amino acids were more effective in reducing liver fat than were similar quantities fed as casein. The lipotropic effect of protein and amino acids is discussed briefly in a review (12) published shortly after the appearance of the paper of Treadwell *et al.*, but at that time no explanation could be offered for these conflicting conclusions.

The data in Table I reveal at least five apparently small, but possibly highly significant differences between the conditions used by Best and Ridout and by Tucker *et al.* It was considered desirable to reinvestigate the matter (a) with a constant nutritional background, (b) at different levels of dietary casein, and (c) to equalize the total nitrogen intake of the rats on the two series of diets (*i.e.*, those containing casein and those containing corresponding amounts of methionine and cystine as free amino acids) by adding to the diets of the latter group a protein known to produce little, if any, lipotropic effect. Gelatin appeared to be suitable for this purpose (3, 13, 14). Further, in order that the diets under comparison might be more nearly alike, it was proposed (d) to add essential amino acids to those of a second group of animals on the diets containing methionine plus gelatin to correct the known deficiencies of these rations. This was considered important, since a preliminary study, suggested by consideration of the data in Table I, had shown that methionine exerts a markedly greater lipotropic effect in the absence (or deficiency) of certain of the essential

amino acids than in their presence (14). Finally, the age of the rat has been shown to influence strongly the deposition of fat in the liver under certain conditions (14-16). This factor differed in the studies mentioned above but was kept constant in the present investigation.

#### EXPERIMENTAL

Fourteen test diets (Series 1 to 5, Table II) were prepared, in each of which the total protein was 35 per cent.<sup>1</sup> Four diets (Series 6), containing 40 per cent protein, and a control diet containing 40 per cent of gelatin, were

TABLE I

*Comparison of Experimental Conditions Used in Previous Attempts to Account for Lipotropic Action of Casein*

		Best <i>et al.</i>	Eckstein <i>et al.</i>
Basal protein		Beef muscle powder, 5%*	Casein, 5%
Supplements compared	Diet A	Casein, 30%	Casein, 15%
	" B	Methionine, 0.96% Cystine, 0.10%	Methionine, 0.465% Cystine, 0.051%
Daily food consumption		8.5 gm.	Not given (assume 8.5 gm.)
" N intake	" A	476 mg.	272 mg.
	" B	76 "	72 "
Essential amino acids	" A	Abundant	Adequate
	" B	Grossly inadequate	Grossly inadequate
Weight of rats, gm.		200	100-120

\* This beef muscle powder, which contained 2.90 per cent methionine and 0.80 per cent cystine, contributed 145 mg. and 44.5 mg., respectively, of these amino acids per 100 gm. to both Diets A and B.

subsequently added. Diet A in each series contained the amount of casein shown in Table II. Diet B in each series contained exactly the same total quantities of methionine and cystine as did the corresponding diets in Group A. The remainder of the nitrogenous moiety of the B diets was supplied by gelatin. Diet C in each series contained total methionine and cystine equivalent to that in Diets A and B, but included also supplements of those essential amino acids which are absent from or markedly deficient in gelatin, but are present in the casein-containing diets.

The scope of the experiment necessitated a compromise in the attempt to balance the sulfur-free essential amino acids in the various diets because

<sup>1</sup> Unpublished work of the authors had shown that in short term experiments the weight of young adult rats could be maintained on a high fat diet (fed *ad libitum*), the protein moiety of which was comprised of 10 per cent casein and 25 per cent gelatin.

of the excessive cost and current limited availability of the amino acids involved. Because 10 per cent of casein in such a diet prevented any weight loss when fed *ad libitum*, supplementary essential amino acids were added to the gelatin diets to make the total amount of each correspond with that in the diet (No. 1A) containing 10 per cent of casein. Additions of *l*(-)-tryptophane, *l*(+)-isoleucine, *dl*-valine, and *dl*-threonine were made, since these are either absent from gelatin or present in quantities very much smaller than in casein. Probably some histidine and phenylalanine should have been included in the supplements, but at the time (1943) they were

TABLE II  
*Supplements Used in Test Diets*

The supplements of casein and amino acids were added at the expense of an equal weight of gelatin. The amounts are given in per cent.

Series No	Group A	Group B		Group C			Group D		
	Casein*	Methi- onine	Cystine	Methi- onine	Cystine	Essential amino acids†	Methi- onine	Cystine	Essential amino acids‡
1	10	0.194	0.045	0.223	0.046	2.84			
2	15	0.291	0.068						
3	20	0.387	0.091	0.417	0.092	2.84			
4	25	0.484	0.113						
5	35	0.677	0.158	0.707	0.160	2.84			
6	40	0.774	0.180	0.803	0.182	2.84	0.871	0.184	9.34

\* Labco casein (2.93 per cent methionine and 0.49 per cent cystine).

† The essential amino acids added to the Group C diets were, in per cent of the diet, *l*(-)-tryptophane 0.22, *l*(+) isoleucine 0.30, *dl*-valine 1.58, *dl* threonine 0.74.

‡ The supplements used in Diet 6D, which was fed at a later date, were based on more recent data and therefore differ slightly from the ratios used in the Group C diets: *l*(-)-tryptophane 0.88, *l*(+)-isoleucine 1.20, *dl*-valine 5.14, and *dl*-threonine 2.12 per cent, respectively.

not available. Because the analytical figures reported from different laboratories often vary considerably, rather arbitrary decisions had to be made as to the values adopted. Table III shows the values chosen and gives an estimate of the amino acid composition of five of the diets used. In Diet 6D not only were the methionine and cystine balanced exactly on Diet 6A, but the essential amino acid supplements were increased correspondingly, thus giving two casein levels (10 and 40 per cent) at which equivalence in this respect was approximated.

The percentage composition of the basal diet (referred to in Table IV as Diet 0) was gelatin<sup>2</sup> 35, beef dripping 40, sucrose 18, salts 5 (McCollum

<sup>2</sup> This gelatin contained 1.02 per cent methionine and 0.01 per cent cystine.



Salt Mixture 185 (38)), agar 2, cod liver oil concentrate<sup>3</sup> 0.015. When Series 6 was later added, a new basal diet was required to control it. This

TABLE III

*Amino Acids in Representative Diets*

Calculated in mg. per 100 gm. of the diet from data in the second and fourth columns and the supplements listed in Table II.\*

Amino acid	Composition assumed				Diet No.				
	Casein		Gelatin		1A	1B	1C	6A	6D
		Bibliographic reference No.		Bibliographic reference No.					
	per cent		per cent						
Glycine.....	0.5 (17)		27.0 (30)		6800	9400	8610	200	7990
Alanine.....	5.5 (18)		9.2 (31)		2850	3200	2930	2220	2720
Serine.....	7.5 (19)		3.3 (32)		1580	1150	1050	3000	980
Threonine.....	3.9 (20)		1.4 (33)		740	490	760	1560	1480
Valine.....	6.3 (21)		2.5 (21)		1260	870	1590	2520	3310
Leucine.....	9.3 (21)		3.3 (21)		1760	1150	1050	3720	980
Isoleucine...	6.1 (21)		1.7 (21)		1040	590	840	2440	1700
Aspartic acid...	6.7 (22)		3.4 (34)		1520	1180	1080	2680	1010
Glutamic "...	24.2 (23)		5.8 (34)		3870	2020	1850	9680	1720
Arginine.....	4.1 (24)		8.9 (24)		2640	3100	2840	1640	2640
Histidine.....	2.4 (25)		0.9 (25)		470	310	290	960	270
Lysine.....	7.3 (25)		5.1 (25)		2000	1770	1620	2420	1510
Cystine.....	0.49 †		0.04 †		57	59	58	196	196
Methionine...	2.93 †		1.0 †		543	542	542	1172	1167
Phenylalanine..	5.2 (25)		2.6 (35)		1170	900	830	2080	770
Tyrosine.....	6.4 (26)		0 (34)		640	0	0	2560	0
Tryptophane....	2.2 (27)		0 (34)		220	0	220	880	880
Proline.....	8.0 (28)		17.5 (36)		5180	6090	5590	3200	5180
Hydroxyproline	0 (28), (29)		14.7 (37)		3680	5110	4690	0	4350

\* For example, the methionine content of the diets is calculated as follows: in Diet 1A 293 mg. from 10 per cent casein plus 250 mg. from 25 per cent gelatin, totaling 543 mg.; in Diet 1B 348 mg. from 34.8 per cent gelatin plus 194 mg. in the supplement, totaling 542 mg.; in Diet 1C 319 mg. from 31.9 per cent gelatin plus 223 mg. in the supplement, totaling 542 mg.

† Unpublished analysis (J. M. R. B.).

diet (referred to as Diet 7) contained gelatin 40 and sucrose 13, but was otherwise the same as Diet 0.

The following B vitamins were injected subcutaneously daily in 0.5 cc. of physiological saline: 25  $\gamma$  of thiamine chloride, 20  $\gamma$  of pyridoxine, 20  $\gamma$  of

<sup>3</sup> Obtained from Ayerst, McKenna and Harrison, Ltd., Montreal. It contains 50,000 I.U. per gm. of vitamin D and 500,000 I.U. per gm. of vitamin A.

riboflavin, 100  $\gamma$  of calcium pantothenate, and 100  $\gamma$  of nicotinic acid. Supplements of casein (Labco, fat-free, vitamin-free), methionine, cystine, and essential amino acids were given at the expense of the gelatin, as described in Table II. All diets within each series were balanced exactly with respect to total methionine and cystine content.

The major dry ingredients of the diets were mixed by hand and the minor ingredients were incorporated as follows: Each one was ground in a mortar

TABLE IV

*Effect of Diets on Liver and Body Weights and on Liver Fat*

Fifteen rats were placed on each diet (except Diets 2A, 2B, 4A, and 4B, for which ten rats were used) for 21 days.

Diet No.	Initial weight, average and range	Change in weight	Food consumed	Weight of moist liver (average)	Crude liver fatty acids (average)
	gm.	per cent	gm. per rat per day	gm.	gm.
0	176 (145-209)	-26.5	7.0	6.33	1.28
1A	183 (116-213)	-5.2	6.9	8.32	2.13
1B	174 (117-215)	-27.2	6.9	5.81	0.73
1C	183 (126-226)	-11.1	7.1	9.73	2.98
2A	182 (117-198)	-6.2	7.1	7.71	1.55
2B	167 (139-230)	-30.5	6.7	5.41	0.50
3A	188 (123-202)	-4.6	7.1	6.63	0.73
3B	175 (138-236)	-27.3	6.8	5.67	0.50
3C	185 (151-213)	-14.1	7.0	6.74	1.11
4A	182 (127-198)	-3.0	7.2	6.63	0.57
4B	164 (149-215)	-24.2	7.0	5.77	0.72
5A	188 (127-207)	+1.3	7.7	7.13	0.51
5B	175 (133-220)	-26.7	7.4	5.72	0.57
5C	184 (134-226)	-7.8	7.2	6.83	0.80
6A	178 (119-247)	+9.3	6.9	7.24	0.48
6B	176 (117-244)	-29.2	6.6	5.38	0.40
6C	181 (112-254)	-7.6	6.4	6.94	0.78
6D	183 (119-254)	-15.6	5.7	6.10	0.59
7	179 (107-235)	-33.2	5.5	4.89	0.50

with a small portion of the dry mixture and the resulting powder was sifted through a 40 mesh screen over the main bulk of the diet, which was then blended thoroughly. The cod liver oil concentrate (dissolved in petroleum ether) was sprayed over the dry ingredients and mixing was continued until the solvent had evaporated. The complete dry mixture was incorporated, with vigorous agitation, into the melted beef dripping, and stirring was continued until the mass solidified. The diets were freshly prepared at about 7 to 10 day intervals and were stored in closed containers in a refrigerator.

Groups of rats (usually fifteen) of the Wistar strain, weighing from 107 to 254 gm., were placed on the test diets for 21 days. The rats in Groups A and C of each series were pair-fed with those of Group B in the same series. Group B was started 3 days ahead of the other groups and 3 day averages were used in determining the food to be given to Groups A and C. Usually an extra 0.5 gm. of the diet was offered daily to each of the pair-fed rats on Diets A and C to correct for the variable amount scattered. The actual average food consumptions are given in Table IV. If one of the control rats (Diet B) died, the corresponding rats on Diets A and C were pair-fed with the rat of closest weight on Diet B. The animals were killed by a blow on the head. Total crude liver fatty acids were determined in the usual way by saponification, acidification, and extraction with petroleum ether (b.p., 30–60°). For brevity the material so obtained is referred to in Table IV as liver fat.

#### DISCUSSION

Attempts to compare the previous studies (8–11, 16) are complicated not only by the difference in the nature of the basal protein used and the different quantities of extra casein added, but also by the different vitamin supplements given (*e.g.*, Best and Ridout supplied only thiamine chloride ("adequate amounts"), whereas Tucker *et al.* supplied the vitamin B complex as yeast tablets). The data here presented, covering the ranges of dietary casein used by the several groups of workers, offer a constant nutritional background with respect to total protein and vitamins, enabling comparisons to be made across the rows and down the columns of Table II.

Fig. 1 shows graphically all the values obtained by the analysis of the individual livers. The variation and the mode are easily seen by inspection, thus making possible a better assessment of the significance of any differences between the mean values reported in Table IV. Four values which were far removed from the others in their respective groups are shown. The failure to join them by a solid line to the other points for the same group indicates that they were not used in calculating the averages given in Table IV. Fig. 2 depicts the mean values obtained for liver fat (total crude fatty acids plus sterols) on the several diets used and shows the relative lipotropic effects over the whole range studied.

The liver fat values of the basal groups (Series 0 and 7) were unexpectedly low in view of the results of Best *et al.* (3) and of Beveridge *et al.* (14), who found that gelatin, when fed at a level of 20 per cent, exerted no demonstrable lipotropic effect. It should be recalled that Series 0 to 5 was run at a different time from Series 6 and 7 and hence any comparisons between them must be made with reservations; nevertheless, the marked decrease in liver fat produced by increasing the dietary gelatin from 35 to 40 per cent is noteworthy. Although gelatin alone (at a 20 per cent dietary level) per-

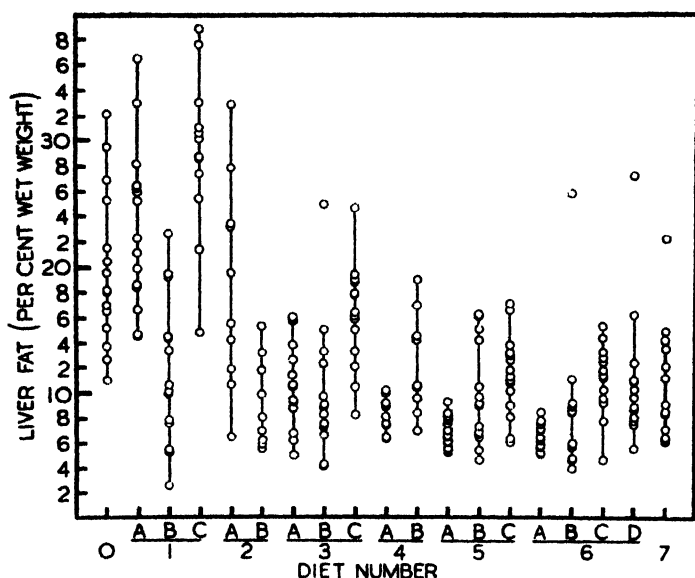


FIG. 1. Total crude fatty acids in individual livers of rats on test diets

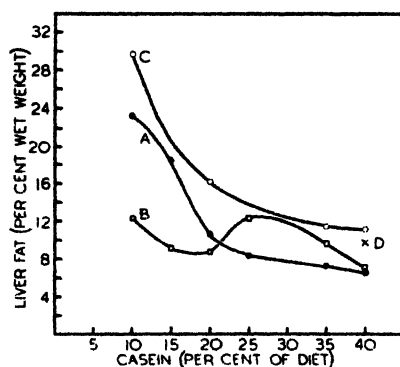


FIG. 2. Effect of various diets on liver fat (mean values for total crude fatty acids expressed as per cent of moist liver weight). Curve A shows the liver fat values obtained on diets containing 35 per cent of total protein (gelatin) with increasing quantities of casein. Curve B shows liver fats on corresponding diets containing the same total protein (supplied as gelatin) with quantities of methionine and cystine added to give exact equivalence at each abscissa. Curve C, same as Curve B, with supplements of essential amino acids, as described in the text. Point D, same as Curve C; see the text.

mitted a large accumulation of fat in the liver, Channon *et al.* (13) have shown that increasing amounts of gelatin added to a diet containing 8 per cent of egg albumin exert a progressive but limited action in decreasing liver fat.

The results on the Group A diets confirm previous reports that increasing the casein content of high fat, low choline diets occasions a progressive decrease in the liver lipids. The decrease is most marked between casein levels of 10 and 20 per cent, but an effect is noted up to the 40 per cent level, although even this quantity of casein does not bring the liver fat down to normal on these diets.

The average liver fat in the rats on the Group B diets is never very high, but the variation on any one diet is considerable, and there is no clear cut progressive decrease in liver fat with increasing dietary methionine, as is the case when comparable quantities of casein are fed (see Fig. 1).

Comparison of the results in Groups A and B reveals that at the 10, 15, and 20 per cent casein levels free methionine causes a greater lowering of liver fat than does a corresponding amount fed in casein (confirming Tucker *et al.*), but that at 25 and 35 per cent casein levels the casein-containing diets produce the greater lipotropic effect (confirming Best and Ridout). The curves in Fig. 2, which intersect at about 22 per cent casein, indicate that at this level and again at about 40 per cent the lipotropic effects of the two types of diet are about equal.

Although Diets A and B in each series were equalized as far as fat, total protein, and methionine and cystine were concerned, the diets of Group A contained reasonable quantities of the essential amino acids (supplied by the casein), while those of Group B lacked tryptophane entirely and were grossly deficient in valine, isoleucine, and threonine. When these defects were approximately corrected in Diets 1C and 6D and partially corrected in the other diets of Group C (in which the essential amino acids were kept constant throughout at levels approximately equivalent to those in the diet containing 10 per cent casein), the addition of increasing amounts of methionine lowered the liver fat progressively, based either on absolute weight of liver fat or on liver fat expressed as per cent of wet weight of the organ. The progressive decrease in liver fat with increasing amounts of the lipotropic factor, noted in the Group A diets but absent from those of Group B, is again clearly demonstrated in the Group C diets.

Comparison of the results of feeding Diet C with those from Diet B of each series reveals that, when a ration lacking or deficient in essential amino acids is supplemented with the quantities of these considered necessary to maintain weight in rats, the lipotropic effects of these diets are minimized and in some cases obliterated.<sup>4</sup> These results, confirming the find-

<sup>4</sup> Similar diets containing 10 per cent casein and 25 per cent gelatin, when fed *ad libitum*, sufficed to prevent loss in weight, as mentioned earlier (foot-note 1). Possibly due to the restricted food intake, the animals on the Group C diets did not maintain their weight, but the extensive losses noted on the Group B diets were considerably reduced.

ings recently published by Beveridge *et al.* (14), emphasize the importance of the nutritional adequacy of diets used in comparative studies of the lipotropic factors.

Increasing the quantity of the essential amino acids from those in a 10 per cent casein diet to those in a 40 per cent casein diet did not affect the liver fat appreciably (compare Diets 6C and 6D, Fig. 1).

Comparison of Curves A and C in Fig. 2 shows that all the groups that received the essential amino acids as supplements to the methionine plus gelatin diets had consistently higher liver fat levels than those on corresponding diets containing casein. These observations lead one to suspect that some lipotropic substance other than methionine occurs in casein, or that the synthetic amino acid preparations were antilipotropic, due either to the presence of some impurity or to the action of the unnatural forms of valine or threonine which were used.

In connection with the first suggestion, it should be noted that in the diets containing casein (Group A) one amino acid is present which is absent from those of Groups B and C. This amino acid is tyrosine. It is of considerable interest, therefore, to recall that Channon *et al.* and Beeston and Platt (9, 39) have suggested the possibility that tyrosine lowers liver lipids. More recently they have reported (40) that a diet containing methionine plus tyrosine gives a greater lipotropic effect than does either one alone. The evidence which they have presented is suggestive but not convincing. However, considering it in conjunction with the data here reported, there is obviously need for clarification of the point. It may be significant, in connection with the above diets, that casein contains a considerable amount of tyrosine, while the gelatin used in the basal ration has none. Experiments to examine the lipotropic action of tyrosine are being undertaken in this laboratory.

Some support for the second possibility appeared in a paper by Albanese and Irby (41), who reported that on a certain diet containing only essential amino acids (supposedly in the proportions found in casein) as the source of nitrogen the growth of young rats was subnormal; the mixture was inferior to a comparable amount of casein, or of casein hydrolyzed by acid or by pancreatic enzymes. They believed there was some evidence that the nutritive inadequacy of the essential amino acid diet may be due, at least in part, to the effects of the unnatural forms of certain amino acids, the non-utilizable enantiomorphs of which were postulated to be toxic. However, Kinsey and Grant (42) in a similar study obtained good growth at almost the same dietary level of total amino acid mixture. Several differences are apparent in the diets used and one or more of these may be of importance to the problem in hand. The fat component of the basal diet of Albanese and Irby consisted of cod liver oil and Crisco, at levels of about 4.5 and 17.5

per cent respectively. Kinsey and Grant fed cod liver oil and corn oil at levels of 2.0 and 10.0 per cent respectively. The former workers fed brewers' yeast; the latter supplied the B vitamins as pure compounds. Further, the ratios of the essential amino acids fed were quite different in the two cases, and it is possible that isoleucine was deficient in the diet used by Albanese and Irby. The more recent findings of Kinsey and Grant, confirming Rose (43), seem to be stronger evidence for the non-toxicity of the unnatural enantiomorphs than does any evidence advanced in support of the idea of a toxic effect. There may be some imbalance of the essential amino acids in our diets, which like those of Albanese and Irby were designed to simulate the ratios in casein. Their diets, like ours, are high fat diets and, like ours, lack tyrosine, which may under these unusual dietary conditions be an important factor. The problems raised by the findings mentioned are of interest and will be investigated.

Beveridge *et al.* (14) and Horning and Eckstein (16) have compared the lipotropic effects of free methionine and equivalent quantities fed as casein in both young and adult rats. They found that free methionine was almost equally effective in reducing liver fat in both age groups, but that casein (at the level fed) was lipotropic in the adult rats only. The probable explanation for these findings is that the amount of protein in the *casein*-supplemented ration supplied enough methionine for maintenance of adult rats and left some over for lipotropic action, but in young rats the quantity of methionine required for growth left little or none for other purposes. However, the diets containing equivalent amounts of *methionine* (fed as free amino acid), being deficient in essential amino acids, did not permit as much growth in young rats and more methionine would thus be left for lipotropic action. This explanation (which is similar to one suggested originally by Griffith and Mulford (44), although theirs is stated in somewhat more general terms) is supported by the data in the present paper as well as by those in several others (11, 14, 16) previously mentioned.

The animals in the experiments here reported were pair-fed and were ingesting not only equal weights of food but equal quantities of protein (or amino acid) nitrogen; yet the casein-fed rats more or less retained their initial weight, or even gained, while those getting only the equivalent amount of methionine (and cystine) lost from 24 to 30 per cent in weight. The deficiencies of essential amino acids appear to have reduced the efficiency (and probably changed the character) of the protein metabolism in such a manner as to leave more dietary methionine free for lipotropic action. In other words, the total nitrogen intake and the adequacy (or inadequacy) of the sulfur-free essential amino acids both play a part in determining the amount of methionine used for general metabolic purposes (*i.e.*, for growth or maintenance), and thus limit the amount available for lipotropic pur-

poses. However, even when the casein-containing and methionine-containing diets were made as nearly identical as possible (*cf.* Diet 1A with Diet 1C and Diet 6A with Diet 6D), there is still a difference in the lipotropic actions of the two diets, indicating the probable existence in casein of an unrecognized factor influencing the deposition of fat in the liver.

The data in Table III may contain a clue as to the nature of this factor. The absence of tyrosine from all diets in Groups C and D has already been commented upon. It should be noted that in general the diets contain adequate amounts of the essential amino acids, as judged by Rose's (45) tentative estimates of the minimal percentages required in a normal diet for optimal growth, *viz.*, threonine 0.6, valine 0.7, leucine 0.9, isoleucine 0.5, arginine 0.2, histidine 0.4, lysine 1.0, methionine 0.6, phenylalanine 0.7, and tryptophane 0.2. In spite of the apparently satisfactory supply of these factors, maintenance of weight was achieved on only two of the diets, Nos. 5A and 6A, containing 35 and 40 per cent respectively, of casein. The high fat content and absence of choline or the restricted food intake may be the cause of the weight losses on the majority of the diets, although some recent work suggests a deleterious effect of gelatin upon growth under somewhat similar conditions (46).

The elegant investigations of Griffith and his collaborators must be mentioned in connection with the explanation offered below for the findings reported in this communication. Griffith and Wade (15) demonstrated that a high fat diet is not necessary for the appearance of fatty livers and renal lesions in young rats and pointed out that the deposition of liver fat is "intensified on those diets permitting the better rates of growth." In 1940 Griffith and Wade (17) fed young rats on a series of low choline, high fat diets containing increasing quantities of casein and noted that the liver fat *increased* as the casein level was raised from 5 to 15 per cent; the incidence of renal lesions continued to increase as the casein level was raised to 25 per cent. Both lesions then decreased in severity as the percentage of casein was further increased, clearly indicating that factors other than the methionine-cystine ratio are involved. Mulford and Griffith (48) showed that the "toxicity" or antilipotropic effect of added cystine decreases as the percentage of casein in the diet increases and disappears at 30 per cent. They also found that the "toxicity" of 0.5 per cent of added cystine in an 18 per cent casein diet is proportional to the food intake, being absent at 2 gm. per day and very severe at 4.6 to 4.9 gm. per day. As early as 1941 Griffith (49) had drawn attention to the fact that the "toxic" effect of cystine is observed only on diets low in cystine and simultaneously low in choline or methionine (*i.e.*, those with a dual deficiency, presumably of organic sulfur compounds and of labile methyl groups). He also pointed out, as had others (9), that the cystine effect is not proportional to the level



of supplementary cystine. Once the sulfur deficiency has been made good, further additions of cystine are without effect. The cystine supplement, by improving growth and stimulating metabolism, brings to light deficiencies previously unrecognized in the diet. With regard to the apparent antilipotropic effect of cystine, Griffith and Mulford (44) have commented that "the deposition of liver fat or the appearance of renal hemorrhage in experiments in which a dietary supplement increases the consumption of food or the rate of growth is not necessarily evidence of a direct antagonism between choline and the dietary supplement."

Thus Griffith has consistently maintained that factors other than the methionine-cystine ratio are important in establishing the lipotropic activity of a diet, and of these other factors he attributes particular importance to the adequacy of the ration.

Most of the facts presented in this report and in the papers referred to seem to be explained by a relatively simple hypothesis; *viz.*, that the amount of dietary methionine available for lipotropic action is limited to that portion not utilized by metabolic processes of apparently higher priority, such as growth or maintenance. The amount required for these non-lipotropic activities is dependent upon the total protein intake and is further modified by the adequacy of the essential amino acids supplied in the diet. If the above hypothesis is accepted, it follows that the *quantity* of any protein fed and the *nature and amount of the sulfur-free essential amino acids* in the protein, as well as its methionine and cystine content, will influence its lipotropic activity because of their effects upon growth and maintenance.

The constant use of one protein (*e.g.*, casein) in the basal diets may obscure the issue or delay the solution of the problem. It was therefore decided to conduct similar experiments with other proteins. Arachin, a globulin from peanuts, which is very low in methionine (50, 51) but otherwise adequate for growth (52), was selected.

Recently a report (53) has appeared which describes the use of arachin in a study of the lipotropic effect of methionine. The data published do not answer all the questions in which we are interested, but they do agree with many of the facts presented here and support the hypothesis presented in this paper concerning the rôle of the essential amino acids.

While the data here reported may not resolve all the anomalies to be noted in previous attempts to explain the varying lipotropic effects of different proteins, they do account for some of the difficulties and point the way for further research.

#### SUMMARY

1. Discrepancies in previous attempts to account for the lipotropic effect of casein, by feeding to one group of rats a certain amount of this protein

and to another group corresponding amounts of methionine and cystine as free amino acids, are accounted for by the finding that different results are obtained at different dietary levels of casein: below 22 per cent the free amino acids exert the stronger effect; above 22 per cent, the casein diet is more lipotropic.

2. The apparently superior lipotropic effect of *free* methionine over an equal quantity *bound* in casein (at casein levels below 22 per cent) is obliterated when the quantities of the essential amino acids in the two diets under comparison are made approximately equal. Thus the lipotropic effect of a diet is determined not only by its content of sulfur-containing amino acids but also by its adequacy in other respects.

3. The lipotropic activity of a protein is determined not only by its methionine and cystine contents, but also by the nature and quantity of the sulfur-free essential amino acids in the protein. These amino acids do not act directly, but through their well known influence on growth and maintenance they influence the formation of new tissue, thus modifying the amount of methionine left available for lipotropic action.

4. Some evidence is presented for the existence in casein of a lipotropic factor other than methionine. Indirect evidence suggests that tyrosine may be involved.

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# RIBONUCLEINASE

## I. MANOMETRIC DETERMINATION OF RIBONUCLEINASE IN BLOOD AND TISSUES OF THE RAT AND THE RABBIT

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The great interest in the enzymes which promote the metabolic changes undergone by the nucleic acids arises from the obvious importance of the latter. For further knowledge of these enzymes convenient methods for their assay are desirable. Such a method has recently been developed by Bain and Rusch (1) for ribonucleinase, the enzyme which catalyzes the depolymerization of ribonucleic acid with the formation of mononucleotides (2). This method has been used in the present studies to assay the blood, blood cells, and plasma, and the spleen, pancreas, and bone marrow of rat and rabbit. Some of the factors affecting the reactivity of the substrate nucleic acid are pointed out. Solubility studies of the nucleic acid before and after being acted on by the enzyme in blood show that the nucleic acid has been depolymerized.

### *Procedure*

*Preparation of Ribonucleinase*—The enzyme for standardizing the test was crystallized twice from ammonium sulfate by the method of Kunitz (3). A solution of the crystals was dialyzed free of ammonium sulfate and the concentration determined from the nitrogen content (3). The stock solution containing 1.90 mg. of enzyme per cc. was stored over chloroform at 7°.

*Preparation of Ribonucleic Acid*—Several commercial brands of yeast nucleic acid were employed. They were purified before use by preparing a 10 per cent solution by neutralization with NaOH, clarifying if necessary by centrifugation or filtration, precipitating with 5 volumes of glacial acetic acid, and washing the precipitate successively with water, 70 per cent ethyl alcohol, 95 per cent alcohol, and ethyl ether. Nucleic acids purified in this manner were usually about twice as reactive with ribonucleinase as were the originals. Precipitation with acetic acid increases the activity of the nucleic acid by reducing the content of mononucleotides which have an inhibitory effect on ribonucleinase (4). In one instance a sample of nucleic acid was encountered which contained copper. The copper accompanied the acetic acid precipitate and was concentrated to such an ex-

tent that it was noticeably inhibitory (4).<sup>1</sup> Any sample of nucleic acid containing mononucleotides<sup>2</sup> whose activity is not improved by acetic acid precipitation should be examined for copper.

*Reactivity of Ribonuclease and Ribonucleic Acid*—The assay method (1) is based on the liberation of CO<sub>2</sub> from a bicarbonate solution by the acidic mononucleotides and intermediate products which are formed by the action of the enzyme (2). The components of the test system were the following: 1.0 cc. of 0.1 M NaHCO<sub>3</sub>, 140 to 185 mg. of nucleic acid in solution at pH 7.5, and appropriate amounts of the crystalline enzyme or the material under test. The total volume of reactants was in most cases 3.5 cc. The usual Warburg equipment was employed. The crystalline enzyme or the nucleic acid was placed in the side arm of the flasks and the system thoroughly gassed at 37° with a 5 per cent CO<sub>2</sub>-95 per cent N<sub>2</sub> mixture. After equilibrium was reached, the reactant in the side arm was tipped in and the manometers read at intervals. The pH attained by this system is 7.5, the optimum for ribonuclease (1, 3).

The relation between the rate of CO<sub>2</sub> evolution, read after the first 5 minutes, and enzyme concentration is shown in Fig. 1. The data were obtained with two different samples of nucleic acid (Samples M<sub>3</sub> and E<sub>1</sub> (4)) after purification. These values have not been corrected for CO<sub>2</sub> retained by the nucleic acid, which would increase them by about 25 per cent. The amounts of the ribonuclease involved in the data given later for blood and tissues can be estimated from Fig. 1.

*Measurement of CO<sub>2</sub> Retention*—In the application of the above test to buffered biological fluids and extracts the CO<sub>2</sub> retained by the buffers must be determined for accurate results. This is most simply done by determining the CO<sub>2</sub> evolved from the buffered system by a standard amount of acid (5). The standard acid (0.30 cc. of 0.01 N citric acid, which liberated 70 c.mm. of CO<sub>2</sub> from an unbuffered system under the same experimental conditions) was put in the side arm of Warburg flasks and dried by being placed in a vacuum desiccator for several hours. Flasks with a second side arm for the nucleic acid were needed but could not be obtained. It was found that flasks with a side arm and without a central well could be adapted for this purpose by placing small cups (to contain 0.5 cc.) made from shell vials in the bottom of the flasks. These cups were very difficult to tip because of capillary attraction between the cups and

<sup>1</sup> Zittle, C. A., unpublished work.

<sup>2</sup> All samples of "nucleic acid" studied have contained a large amount of mononucleotides (4); however, several preparations of "sodium nucleinate" that have been tested have had initially excellent reactivity with ribonuclease. Apparently the method of manufacture of the latter substances gives a product of low mononucleotide content.

the wet surfaces of the flasks. Tipping was facilitated by placing a glass rod approximately 2.5 mm. in diameter on the bottom of the flask over which the cup could be tipped. The volume occupied by the cup and rod (maximum total 0.80 cc.) was determined in a wide mouthed pycnometer

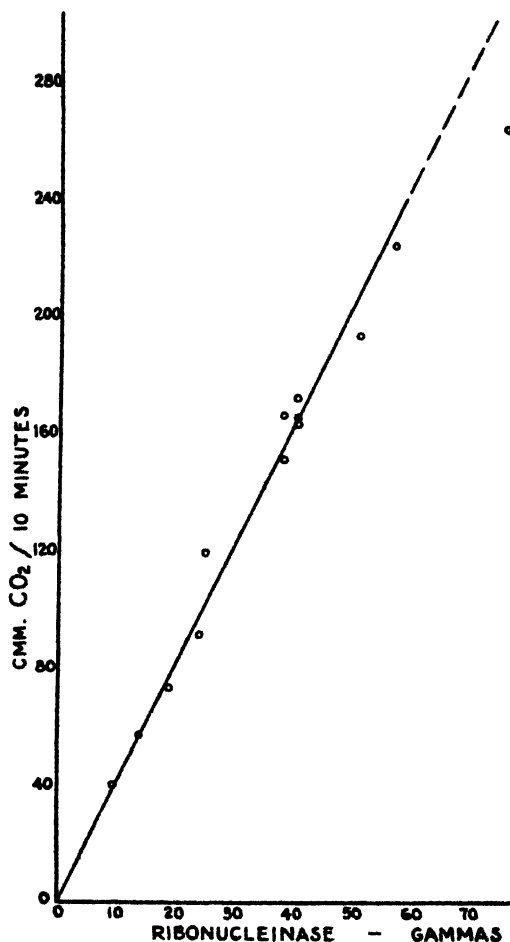


FIG. 1. Rate of carbon dioxide evolution in relation to concentration of ribonucleinase. 185 mg. of nucleic acid were used as the substrate.

and the flask constants recalculated. The nucleic acid (0.5 cc. of about 30 per cent concentration) was placed in the cup and the main part of the flask contained the fluid to be analyzed, the  $\text{NaHCO}_3$  buffer, and saline to make a volume of 3.5 cc.

After the usual equilibration was performed, the substrate was tipped

in,<sup>3</sup> the manometer read at intervals, and finally the citric acid added, and readings taken until a steady rate was reached. Data obtained in this manner were procured for nucleic acid alone and for rabbit and rat blood, plasma, and tissue extracts with the substrate nucleic acid present. The magnitude of the retention correction is shown by the following data calculated with the usual flask constants: gas liberated from unbuffered system, 70 c.mm.; with 185 mg. of nucleic acid, 56 c.mm.; with 1.0 cc. of plasma, 50 c.mm.; with 1.0 cc. of blood cells (diluted with 0.85 per cent NaCl to the volume of blood from which they were taken), 34 c.mm.; with 1.0 cc. of blood, 33 c.mm. Flask constants which correct for this retention are calculated from the ratio of the CO<sub>2</sub> released from the unbuffered system and the manometer readings obtained with the buffered test systems. The data obtained with individual flasks were calculated for the use of all the flasks, which ranged from 14.0 to 15.6 cc. in total volume, by the method of Bain and Rusch (1). The constants were plotted against the total volume of the flasks instead of the gas volume used by these authors. The constants for any fluid volume were readily obtained from a curve relating the flask constants and the fluid volume (6). Variation in the buffering capacity (protein, cells, etc.) of the blood would be reflected in changes in the retention constants; however, for many purposes average values are satisfactory.

*Assay of Blood and Tissues*—In performing the assays a control flask was prepared to which no nucleic acid was added. With whole blood the release of CO<sub>2</sub>, probably due to glycolysis, was on the average 72 c.mm. per 1.0 cc. per hour; with cells this correction was about half as great and with plasma negligible.

The results of the assays for rat and rabbit shown in Table I are in general parallel.

In order to be sure that the gas liberated from the reaction of the blood and nucleic acid represented a breakdown of the nucleic acid (2, 3), the increase in the acid solubility of the substrate nucleic acid was studied. The usual analytical experiment was set up with rat blood (1.0 cc. of 0.1 M NaHCO<sub>3</sub>, 0.5 cc. of 30 per cent nucleic acid, 1.0 or 2.0 cc. of blood, and 0.85 per cent NaCl to make 3.5 cc.). The activity was 106 c.mm. per 1.0 cc. per hour, after correction for the metabolism of the control of 105 c.mm. per 1.0 cc. per hour. The experiment was continued for 2.2 hours, 0.5 cc. of 30 per cent nucleic acid was then added to the control flasks, and

<sup>3</sup> The capacity of the cup or of the side arm was not sufficient to hold the amount of blood required for the best performance of the test. Also in instances in which the blood was added from the side arm the large amount of O<sub>2</sub> evolved from the hemoglobin in the presence of the NaHCO<sub>3</sub> left little room remaining in the manometer for the assay.

all were immediately diluted to contain 10 mg. of nucleic acid per cc. and precipitated with an equal volume of 0.64 N trichloroacetic acid.<sup>4</sup> The temperature of the mixtures was 24° (7). The mixtures were centrifuged

TABLE I

*Ribonucleinase Activity of Blood, Plasma, and Blood Cells of Rat and Rabbit*

Blood obtained by heart puncture from adult animals was collected in a tube containing 1.0 to 2.0 cc. of 2 per cent sodium oxalate to 10 cc. of blood. The oxalate did not affect the results with crystalline ribonucleinase. A precipitate, probably calcium oxalate, formed with the nucleic acid. Usually the blood from two or three rats was pooled. The blood cells were diluted with 0.85 per cent NaCl to the volume of the blood from which they were taken.

The results are expressed in c.m.m. per cc. per hour.

	Blood			Plasma			Cells		
	No. of samples	Range	Average	No. of samples	Range	Average	No. of samples	Range	Average
Rat...	5	84-204	126	8	30-84	42	5	36-84	60
Rabbit...	3	24-144	78	2	8-25	16	3	36-96	66

TABLE II

*Ribonucleinase Activity of Tissues from Rat and Rabbit*

The bone marrow was obtained from the bones of the hind legs. In the case of the rabbit a 0.5 gm. sample was removed from the end portion of the femurs. The pancreatic tissue was difficult to sample, especially so from the rabbit. The minced tissue was suspended in water (100 mg. per 1.0 cc.) and broken up in a homogenizer (8). Treatment in a sonic oscillator, frequency about 10,000 cycles per second, for 5 minutes effectively dispersed the bone marrow but was not satisfactory for the other tissues.

The results are expressed in c.m.m. per mg (wet weight) per hour.

	Bone marrow			Spleen			Pancreas		
	No. of samples	Range	Average	No. of samples	Range	Average	No. of samples	Range	Average
Rat .....	2	4.1-6.6	5.4	4	3.5-5.5	4.5	4	9.9-27.2	17.0
Rabbit...	8	0.8-2.4	1.5	6	2.9-9.0	4.7	3	0.6- 3.2	2.4

for 10 minutes and the supernatant fluids analyzed for phosphorus. The control solution was found to contain 0.090 mg. of acid-soluble phosphorus

<sup>4</sup> Preliminary experiments had shown that the concentration of trichloroacetic acid used gave the same final pH and precipitated the nucleic acid as effectively as 0.5 N HCl (7). This acid was preferred to HCl because it precipitated the blood proteins as well as the undecomposed nucleic acid.



per cc. (total phosphorus = 0.391 mg. per cc.), whereas the solution digested with blood for 2.2 hours contained 0.172 mg. of phosphorus per cc., representing a 27 per cent increase in the solubility of the nucleic acid per cc. of blood. Thus the breakdown of nucleic acid, assumed to be the cause of the evolution of  $\text{CO}_2$ , had taken place as a result of the action of the blood. Under the same conditions 4  $\gamma$  of crystalline ribonucleinase were required to produce a similar increase in the solubility of the nucleic acid.

The ribonucleinase activity found in some of the tissues of the rat and rabbit is shown in Table II.

#### DISCUSSION

The evolution of  $\text{CO}_2$  obtained per unit of enzyme (Fig. 1) is about 50 per cent greater than the results reported by Bain and Rusch (1). This may be due to a difference in the substrate nucleic acid (4), although other factors may be responsible. Both sets of data should be increased about 25 per cent for the  $\text{CO}_2$  retention by the substrate nucleic acid, an effect that apparently was not considered in the experiments of Bain and Rusch with the pure ribonucleinase (1).

A significant amount of ribonucleinase is present in the blood of rat and rabbit, largely in the cellular portion. The approximately 3-fold variation in the assay values for a given tissue represents an animal difference, an explanation of which is not yet apparent. Bain and Rusch (1) stated that ribonucleinase was present in whole blood of the rat but they gave no quantitative results. These authors did report the ribonucleinase content of various rat tissues. They found the activity of pancreas to be 16.7 c.mm. per mg. per hour (range, 13.0 to 20.0, eight samples), of spleen to be 2.06 (range, 1.80 to 2.49, twelve samples). The results shown in Table II for the latter tissue are somewhat higher. The ribonucleinase in the bone marrow and pancreas of the rat is significantly higher than that from the same tissues of the rabbit. Since the range of normal values has been established, the assay method will be used to study various induced pathological states in rabbit and rat which may reveal the relation of the changes caused by this enzyme to other vital processes.

#### SUMMARY

The ribonucleinase contents of the blood, plasma, and blood cells of the rat and the rabbit were determined. Most of the enzyme was found to be in the blood cells. Assays were also performed on the bone marrow, spleen, and pancreas of the rat and the rabbit. Solubility studies yielded confirming evidence that the nucleic acid is broken down by the blood.

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# RIBONUCLEINASE

## II. MONONUCLEOTIDES IN COMMERCIAL RIBONUCLEIC ACIDS AND THEIR EFFECT ON RIBONUCLEINASE

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Nucleic acids intended for use as a substrate for ribonucleinase have been purified by precipitation with glacial acetic acid (1-3), a procedure that is reported (3) to be essential for optimum reactivity of substrate and enzyme. It is suggested (3) that the poor results with the unpurified nucleic acid may be due to "decomposition products present in the crude nucleic acid."

Since many enzymes are inhibited by the products of their action, the effect of mononucleotides on ribonucleinase is of interest because they are formed when the enzyme acts on ribonucleic acid (4). The present studies have shown that the mononucleotides are inhibitory to ribonucleinase and that they are present in commercial nucleic acids prepared as the free acids, whereas the sodium nucleinate preparations are relatively free of the mononucleotides. A corresponding difference in the reactivity of the two types of preparation with ribonucleinase is encountered.

### *Procedure*

The activity of ribonucleinase was determined by the manometric method recently developed by Bain and Rusch (3). The enzyme was twice crystallized and the nucleic acid was purified by precipitation with acetic acid (5).

*Effect of Purification on Reactivity of Ribonucleic Acids*—The reactivities of the two types of commercial nucleic acids (free acid and sodium salt) with ribonucleinase before and after precipitation with glacial acetic acid are shown in Table I. The sodium nucleinate preparations had initially a high activity, compared with that of the free nucleic acid preparations, and precipitation with acetic acid, in the one instance that it was performed, did not increase the activity. All the free nucleic acid samples, however, with one exception, increased in reactivity 33 to 85 per cent on precipitation.

The data in Table II show that the difference in behavior of the two types of preparation can be correlated with the content of mononucleotides

(solubility in the uranium chloride reagent) (6) and the less reactive<sup>1</sup> low polymer fraction (solubility in HCl and in acetic acid).

The exceptional nucleic acid whose reactivity was not increased by precipitation with acetic acid was Sample S. This nucleic acid gave poor yields on precipitation with acetic acid, indicative of a high content of the soluble mono- and tetranucleotides, and it was supposed that the precipitated material still contained mononucleotides which are shown later to be

TABLE I  
*Activity of Ribonuclease with Unpurified and Purified Nucleic Acids*

Sample*	Type of preparation†	Activity		Increase in activity from purification
		Unpurified	Purified	
		<i>c mm per γ per hr.</i>	<i>c mm. per γ per hr.</i>	<i>per cent</i>
P	Free acid	14.5	26.9	85
EA <sub>3</sub>	" "	18.5	28.1	52
M <sub>3</sub>	" "	16.8	23.4	39
E <sub>1</sub>	" "	16.1	24.0	49
E <sub>2</sub>	" "	14.2	18.9	33
S	" "	15.2	14.0	0
EA <sub>1</sub>	Sodium salt	30.6		
EA <sub>2</sub>	" "	27.6	25.6	0
M <sub>1</sub>	" "	22.8		
M <sub>2</sub>	" "	19.7		

\* These samples were obtained commercially: P, Pfanstiehl; M, Merck; E, Eastman; S, Schwarz; EA, Eimer and Amend.

† Both materials when tested were in the form of sodium salts, since solutions brought to pH 7.5 with NaOH were used. For these tests the side arm of Warburg flasks contained 19 γ or 38 γ of ribonuclease (the activity was proportional to the amount of enzyme over this range; see Fig. 1 of Paper I); the main part of the flask contained 1.0 cc. of 0.1 M NaHCO<sub>3</sub>, 185 mg. of nucleic acid in solution at pH 7.5, and water or 0.85 per cent NaCl to make the total volume after mixing 3.5 cc. NaCl in this concentration had no effect on the enzyme. The flasks were equilibrated at 37° with a mixture of 5 per cent CO<sub>2</sub>-95 per cent N<sub>2</sub>. The enzyme was tipped in and readings taken at intervals and those between 5 and 20 minutes used for calculating the activities.

inhibitory; subsequent experiments showed that this was not the cause of the poor activity. Data which might throw light on this question are presented in Table II. Study of the solubilities of the crude and purified nucleic acid in HCl and in the uranium chloride reagent showed that

<sup>1</sup> It is expected that weight for weight a tetranucleotide would be less reactive than a polynucleotide, since the former would contain three bonds that could be opened by the enzyme, whereas the same bonds in the latter would approach four as a maximum.

nucleotides were present but did not explain the unusual behavior of purified Sample S. Since the recovery with glacial acetic acid was exception-

TABLE II  
*Solubility of Nucleic Acids in Various Acids*

Sample	Type of preparation*	Solubility in uranium chloride reagent†	Solubility in 0.25 N HCl‡	Solubility in acetic acid	Material lost in purifying with acetic acid§
		per cent	per cent	per cent	per cent
P, crude	Free acid	15.1	53.2		
" purified		3.8	17.6	19.1	60
M <sub>1</sub> , crude	" "		45.3		
" purified			13.1		55
S, crude	" "	7.5	51.2		
" purified		4.6	17.8	28.9	78
E <sub>1</sub> , crude	" "	11.6			
" purified		1.4			36
E <sub>2</sub> , crude	" "				
EA <sub>3</sub> , crude	" "	5.1	19.3	45.1	75
" purified		1.0	9.2	20.1	46
EA <sub>2</sub> , crude	Sodium salt	2.4	40.1	28.5	
" purified		0.9		12.9	19
M <sub>1</sub> , crude	" "	6.1	31.3	26.9	

The general procedure for determining the solubility was as follows: A 1 per cent solution of nucleic acid which had been adjusted with NaOH to pH 7.0 was precipitated with an equal volume of the uranium chloride reagent or 0.5 N HCl or 5 volumes of glacial acetic acid. The temperature was 23° (2). The last two were centrifuged immediately, the first after a lapse of 30 minutes (1). The nucleic acid solubility was based on the phosphorus in the original solution and in the supernatant fluids from the precipitate. The inorganic phosphorus in these preparations was inappreciable; in crude Sample P for example, only 0.37 per cent of the total phosphorus was inorganic, and this was only 4.8 per cent of the phosphorus soluble in the uranium reagent.

\* All preparations when tested were in the form of sodium salts.

† 0.25 per cent uranium chloride in 2.5 per cent trichloroacetic acid (1).

‡ The procedure described by Woodward (2) was followed.

§ The purification was accomplished as described (5) by precipitation with 5 volumes of glacial acetic acid and washing the precipitate with ethyl alcohol and ether.

|| These experiments were not very satisfactory because of the colloidal nature of the precipitate which made a clean separation of sediment and supernatant fluid difficult. All precipitates in HCl were colloidal to some degree, whereas the precipitates in both acetic acid and the uranium chloride reagent were gelatinous and readily sedimented in the centrifuge.

ally poor, the behavior of purified Samples P and S with this reagent was compared. The solubility of purified Sample S in glacial acetic acid was somewhat high but not enough to explain its low activity. Eventually it

was found that crude Sample S contained copper which was sufficiently concentrated by the precipitation with acetic acid to exert an inhibitory effect.<sup>2</sup>

*Effect of Mononucleotides on Ribonucleinase Activity*—Since the acid-soluble fraction of nucleic acid (mono- or tetranucleotides) appeared to be largely responsible for the poor activity of the crude nucleic acids, mono-

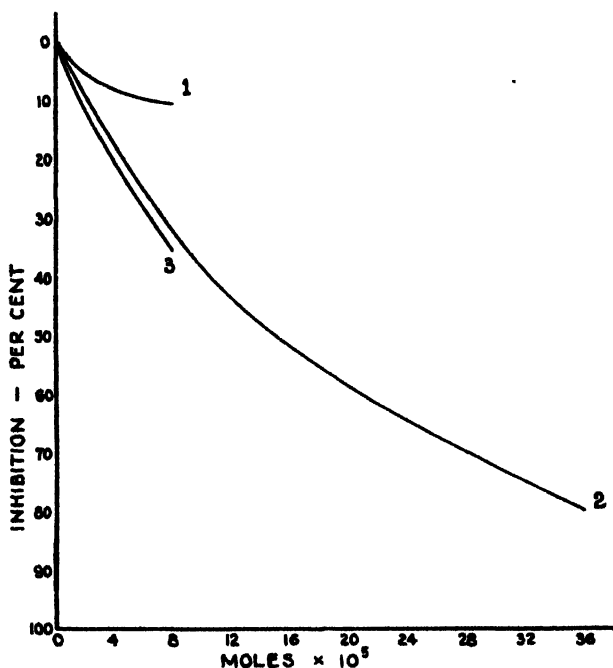


FIG. 1. Effect of mononucleotides on ribonucleinase. The number of moles indicated was contained in the test volume of 3.5 cc., with 140 mg. of nucleic acid present. Curve 1, adenylic acid; Curve 2, mixture of mononucleotides; Curve 3, guanylic acid. The curves were calculated from smooth curves through experimental points and the  $\text{CO}_2$  retention curves for the same concentration range of mononucleotides.

nucleotides were added to the test system containing the purified nucleic acid. The solution of mononucleotides was prepared by alkaline hydrolysis of purified nucleic acid<sup>3</sup> and neutralization with acetic acid. The inhibitory

<sup>2</sup> Zittle, C. A., unpublished work.

<sup>3</sup> The mononucleotides were prepared by hydrolysis of the nucleic acid with NaOH at room temperature for 24 hours (7). The hydrolysate was used after neutralization with acetic acid and removal of a variable amount of flocculent precipitate which appeared on neutralization.

effect of this solution on ribonucleinase is shown in Fig. 1 where the moles<sup>4</sup> of substance added are plotted against the percentage of inhibition. The data have been corrected for CO<sub>2</sub> retained (5) by the buffering action of the substances under study and it was found that sodium acetate in excess of the amount in the mononucleotides had no effect on the ribonucleinase. Purified adenylic and guanylic acids were inhibitory (Fig. 1), as would be expected since they are component nucleotides of ribonucleic acid.

#### DISCUSSION

All the commercial nucleic acids (free acid) in the crude state had about the same reactivity with ribonucleinase but there was greater variability in their behavior after purification. In one instance in which no increase in reactivity was obtained the presence of copper was found to be the cause (5).<sup>2</sup> This may be a contributing factor in the reactivity of some of the other samples but the predominant effect is that due to the inhibitory mononucleotides and the less reactive tetranucleotides. Small differences in activity, shown in Table I, cannot be explained by the data in Table II. The precipitations by HCl and by acetic acid are influenced by salts (1), and inhibitory substances other than copper may affect the reactivity of the enzyme; however, in general, when the copper content is low, solubility in these reagents will give a good measure of the reactivity with ribonucleinase to be expected. Unfortunately products from the same manufacturer are not always uniform. This is brought out by a comparison of Samples E<sub>1</sub> and E<sub>2</sub> in regard to material lost in purification (Table II) and reactivity before and after purification (Table I).

The nucleic acids prepared commercially as the sodium salts have consistently been more active than those supplied as the free acids and are about as reactive as the latter after purification. Apparently the method of manufacture of the sodium salt gives a product of low nucleotide content and this is confirmed by the solubility data. The superior quality of purified Samples EA<sub>2</sub> and EA<sub>3</sub> may be due to a higher degree of polymerization, as well as low mononucleotide content, for their solutions were very viscous and jelled in a 30 per cent solution at 7°.

The effect of mononucleotides on ribonucleinase is probably a competitive inhibition, an interpretation which is supported by the shape of the inhibition curves. The hyperbolic type of inhibition-concentration curve is encountered when an enzyme is inhibited by the formation of a *dissociable* inactive compound with the inhibiting substance and is characteristic of competitive inhibition. This type of curve is well illustrated by the inhibition of invertase by monosaccharides (8) and the inhibition of cholin-

<sup>4</sup> The number of moles in the solution of mixed nucleotides was calculated from the average molecular weight of the four mononucleotides.



esterase by prostigmine (9). These inhibition curves are rectangular hyperbolas described by the following equation,  $E'/E = 1/1 + I/k$  (8), where  $E$  is the activity of the enzyme without inhibitor,  $E'$  the activity with it,  $I$  the concentration of inhibitor, and  $k$  the dissociation constant of the inactive inhibitor-enzyme combination. This can be put in the form  $(E - E')k = E'I$ , for determination of  $k$  by plotting  $E - E'$  versus  $E'I$ . The data for guanylic and adenylic acids as well as the data for the mixture of mononucleotides up to a concentration of  $12 \times 10^{-5}$  mole are in fair agreement with this equation.

An added compound may competitively affect the enzyme activity in two different ways (8): the activity may be equally reduced at all substrate concentrations, in which case, presumably, the inhibiting substance united with the enzyme is not displaced from it by the substrate, or in the other case, the inhibiting substance produces a far greater effect in weak than in strong substrate solutions. The effect of mononucleotides is of the first type. The data recorded in Fig. 1 are for 140 mg. of nucleic acid; the percentage inhibition, however, with 92 mg. of nucleic acid is about the same. With the mononucleotide mixture, for example, at  $24.6 \times 10^{-5}$  mole, the inhibition was 65 per cent for the higher concentration of nucleic acid compared with 69 per cent for the lower. Also with  $9.0 \times 10^{-5}$  mole of guanylic acid the fractional activity was about the same for the two concentrations of nucleic acid.

Kunitz (1) has observed that even with maximum digestion of nucleic acid only 40 per cent of it had become soluble in the uranium chloride reagent; *i.e.*, had been broken down to mononucleotides. This observation has been confirmed in these studies and it was thought that the inhibitory effect of mononucleotides might be the explanation. Experiments which will be reported later have shown that this cannot be the explanation of the abrupt termination of the digestion at the 30 to 40 per cent level.

#### SUMMARY

The difference in reactivity between the two types of commercial nucleic acid preparations (free acid and sodium salt) with ribonucleinase has been correlated with the difference in mono- and tetranucleotide content. The increased reactivity of the free acid with ribonucleinase after precipitation with acetic acid has been ascribed to the removal of these substances, since ribonucleinase was inhibited by a mixture of mononucleotides, by adenylic and by guanylic acids, and since the tetranucleotides are expected to be less reactive than a polynucleotide. The inhibition of ribonucleinase by mononucleotides was probably due to the competition of the mononucleotides with the substrate for the enzyme. This inhibition was about the same with several different concentrations of nucleic acid.

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# THE COLORIMETRIC ASSAY OF URINARY CORTICOSTEROID-LIKE SUBSTANCES\*

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It has been demonstrated that human urine contains substances which resemble both in their chemical characteristics and biologic actions certain of the 11-oxycorticosteroids which have been isolated from the adrenal cortex of animals (1-10). It has also been shown by biologic assays that these substances are excreted in increased quantities by postoperative, burned, or otherwise damaged persons (3, 8, 9). These findings appear to substantiate the thesis that the adrenal cortex plays a rôle in the defense of the organism against such stresses (11-13).

The present paper reports investigations on the urinary corticosteroid output by normal and abnormal subjects as measured by a colorimetric assay procedure which is described here. With the exception of the final colorimetric determination, the analytic procedure used closely resembles that described by Venning, Hoffman, and Browne (9). A method for the colorimetric assay of glucose (14) was adapted to meet the requirements for measuring corticosteroids.

*Reagents*—All reagents are of C.P. quality. All apparatus is of glass. Stop-cocks are lubricated only with water. The chloroform, ethylene dichloride, ethyl ether, and benzene used are purified by distillation in an all-glass distillation apparatus. The head and tail fractions of the distillate are discarded. The middle fraction of the distillate is tested for the presence of reducing agents as follows: A 150 cc. aliquot is evaporated to dryness. The residue is dissolved in 2 cc. of methanol. Of this solution 0.5 cc. is assayed for reducing agents according to the analytic procedure. The galvanometer reading obtained should be 99 to 100. The reagents used in the colorimetric assay are prepared according to the directions of Nelson (14). Stock Copper Solution A is stored in a dark Pyrex bottle and is freshly prepared at 2 week intervals.

## *Analytic Procedure*

*Step 1. Collection and Extraction of Urine*—A 24 hour sample of urine is collected without preservative. The urine is stored in a cool place during

\* This work was supported by a grant from the Commonwealth Fund.

collection and prior to extraction. Preferably within 1 or 2 days after the completion of the collection period, the urine is extracted by shaking four times with 15 volumes per cent of chloroform in a separatory funnel. The total quantity of chloroform used is recorded. 12 instead of 24 hour samples of urine may be used for patients who are excreting normal or excessively high quantities of urinary corticosteroids. The combined chloroform extract is often emulsified. Such emulsions are partially separated from the chloroform by centrifugation. That aliquot of the total chloroform which has been thus freed from emulsions is accurately measured so that losses of chloroform (and hence of urinary steroid) in the emulsion may be estimated and a correction factor applied. The chloroform extract thus measured is evaporated to dryness in a vacuum distillation apparatus at a temperature not exceeding 50°. The dry residue may be stored in the refrigerator for 24 hours without appreciable losses of steroid.

*Step 2. Purification of Crude Chloroform Extract*—The chloroform residue is transferred with 100 cc. of chloroform to a separatory funnel. The solution is then washed three times with 10 cc. of chilled 0.1 N aqueous NaOH solution and three times with 10 cc. lots of water. Each wash is extracted back with 10 cc. of chloroform which is added to the original chloroform solution before the next NaOH or water wash is carried out. The NaOH and water washings are discarded. The washed chloroform solution is evaporated to dryness at a temperature not exceeding 50°.

*Step 3. Partition between Benzene and Water*—The chloroform residue is transferred quantitatively to a small separatory funnel with a total of 30 cc. of benzene. The benzene solution is extracted ten times with 30 cc. lots of water. The benzene is then discarded. The combined aqueous extract is transferred to a separatory funnel where it is extracted four times with 45 cc. lots of chloroform. The chloroform extracts are combined and evaporated to dryness at a temperature not exceeding 50° (*crude fraction*).

This crude fraction may be measured for reducing agents according to the colorimetric assay procedure given below. Prior to such assay, remaining traces of chloroform must be removed from the residue by the addition and subsequent evaporation at 50° of three 5 cc. lots of methanol. It has been customary to dissolve the dried residue in 5 cc. of methanol and to take 0.5 cc. of this solution of crude fraction for colorimetric assay. The remaining 4.5 cc. of the methanol solution are evaporated to dryness and the residue further purified by treatment with Girard's Reagent T.

*Step 4. Separation of Crude Extract into Ketonic and Non-Ketonic Fractions by Treatment with Girard's Reagent T*—To the dried residue of the crude fraction, 200 mg. of Girard's Reagent T (trimethylacetylhydrazide-ammonium chloride, Eastman Kodak Company) and 0.5 cc. of glacial

acetic acid are added. The flask is stoppered with tin-foil or aluminum foil. The mixture is then heated for 2 minutes in a boiling water bath. During this period the flask is rotated to insure complete mixing of the contents. At the end of this period the flask is transferred from the boiling water bath to an ice bath. After the contents have become chilled, they are transferred quantitatively with 40 cc. of ice-cold distilled water to a small separatory funnel. 3 cc. of 10 per cent aqueous sodium hydroxide solution are added. The mixture is then extracted three times with 20 cc. portions of chloroform. The chloroform extracts are combined and washed once with 60 cc. of distilled water. This chloroform extract (non-ketonic fraction) is discarded. 1 cc. of concentrated sulfuric acid is added to the foregoing water washing, which is then added to the aqueous phase remaining after chloroform extraction of the non-ketonic fraction. After the addition of 20 cc. of chloroform, the resultant mixture is allowed to stand at room temperature for approximately 2 hours. It is then extracted with the chloroform already present and with three additional 20 cc. lots of chloroform. The combined chloroform extract (ketonic fraction) is washed once with 10 cc. of 0.1 N sodium hydroxide solution and three times with 10 cc. lots of water. Each wash is extracted back with an equal volume of chloroform which is added to the original chloroform solution before the next wash is carried out. The washed chloroform extract is evaporated to dryness at a temperature not exceeding 50°. The residue is freed from chloroform by the addition and subsequent evaporation of three 5 cc. lots of methanol. This is necessary because traces of chloroform interfere with the colorimetric assay. The dried residue (*ketonic residue*) is dissolved in a measured quantity of methanol. An aliquot of this methanol solution is assayed according to the colorimetric assay procedure given below. With extracts of urine from normal patients it is usually convenient to use a total of 2 cc. of methanol for dissolving this residue and to take 0.5 cc. of the solution for colorimetric assay. For patients with abnormally high assay values smaller aliquots may be used.

*Colorimetric Assay* - 0.5 cc. of a methanol solution of corticosteroid and 0.5 cc. of water are delivered near the bottom of a Folin-Wu macro blood sugar tube calibrated at 7.0 cc. 1 cc. of copper reagent (prepared fresh just before use by mixing 25 parts of Copper Reagent A and 1 part of Copper Reagent B according to directions given elsewhere (14)) is then delivered near the bottom of the tube. The contents of the tube are mixed by gentle shaking and are heated in a boiling water bath for 20 minutes. The tube is then cooled under a stream of tap water and 1 cc. of arsenomolybdate reagent (14) is added. The contents of the tube are again mixed by gentle agitation. Water is added to make a total volume of 7 cc. The tube is stoppered with the hand and after the contents have been mixed by in-

verting the tube, they are transferred to a photoelectric colorimeter tube.<sup>1</sup> A blank prepared in the same way but containing no steroid is developed in the same manner. By means of a filter with maximum transmission at 660 m $\mu$  the galvanometer is adjusted to 100 with a tube containing the blank in place. This tube is then replaced by that containing the unknown sample and the galvanometer reading is recorded. The quantity of "corticosteroid" present is calculated from this galvanometer reading by referring to a calibration curve in which galvanometer readings are plotted against known amounts of corticosteroid.

#### EXPERIMENTAL

*Determination by Colorimetric Assay of Crystalline Corticosteroids in Pure Solution*—Table I gives representative values obtained in the course of establishing proportionality constants ( $K$ ) between the quantity of crystalline corticosteroid<sup>2</sup> in the sample analyzed ( $C$ ) and the amount shown by the galvanometer reading ( $G$ ) when  $C = 1/K (2 - \log G)$ . It is seen that for 17-OH corticosterone, corticosterone, and dehydrocorticosterone in amounts ranging between approximately 0.03 and 0.08 mg.  $K$  is equal to  $5.4 \pm 0.1$ .<sup>3</sup> With smaller amounts of these steroids, lower  $K$  values were obtained. On the other hand, for from 0.04 to 0.07 mg. of 17-OH dehydrocorticosterone,  $K$  is approximately 3.9. It is not known whether this discrepancy is real or is due to deterioration of the sample of 17-OH dehydrocorticosterone used.<sup>4</sup> Until another sample of this steroid becomes available for study, it will be assumed that a calibration curve described by the  $K$  value 5.4 may be applied with reasonable accuracy in the measurement of all four of these corticosteroids and of similar substances extracted from urine by the analytic procedure described above.

*Partition of Crystalline Corticosteroids between Water and Various Organic Solvents*—In these experiments approximately 0.2 mg. of 17-OH corticosterone, 17-OH dehydrocorticosterone, corticosterone, or dehydrocorticosterone was dissolved in 50 cc. of water. The aqueous solution was quantitatively transferred to a separatory funnel with an equal volume of ethylene dichloride, chloroform, or ethyl ether. The funnel was shaken

<sup>1</sup> An Evelyn macro photoelectric colorimeter was used in this laboratory.

<sup>2</sup> We are greatly indebted to Professor E. C. Kendall for crystalline samples of Compound A (dehydrocorticosterone, m.p. 177–180.5°), Compound B (corticosterone, m.p. 177–179°), and Compound E (17-OH dehydrocorticosterone m.p., 224–225°), to Dr. G. Thorn for a sample of crystalline 17-OH corticosterone, and to Dr. E. Schwenk of the Schering Corporation for a sample of crystalline desoxycorticosterone.

<sup>3</sup> In this reaction 1 mg. of 17-OH corticosterone, corticosterone, or dehydrocorticosterone, respectively, is equal to approximately 0.1 mg. of glucose.

<sup>4</sup> This discrepancy is not explained by differences in the molecular weights of the four corticosteroids.

vigorously for 3 minutes and then allowed to stand until the aqueous and organic solvent phases had separated completely. The organic solvent phase was removed, evaporated to dryness at 50°, and the corticosteroid content of the residue determined by the colorimetric assay procedure.

Chloroform extracted each of the respective corticosteroids almost quantitatively (average, 102 per cent). Ethylene dichloride was slightly less efficient (average, 92 per cent), while ethyl ether proved to be a considerably less efficient solvent, especially for the two 17-OH steroids (17-OH corticosterone, 65 per cent; 17-OH dehydrocorticosterone, 51 per cent).

TABLE I

*Experiments Taken from Series Establishing Proportionality Constant (K) between Total Crystalline Corticosteroid Determined in Sample and Amount Shown by Galvanometer Reading*

Corticosteroid	Total corticosteroid in sample analyzed	Galvanometer reading	K
	mg.		
17-OH corticosterone	0.010	91	4.1
	0.030	69 <sup>2</sup>	5.3
	0.050	54 <sup>2</sup>	5.3
	0.076	39 <sup>2</sup>	5.3
17-OH dehydrocorticosterone	0.015	90	3.1
	0.044	68 <sup>2</sup>	3.7
	0.074	50	4.1
Corticosterone	0.010	88	5.3
	0.031	67 <sup>2</sup>	5.5
	0.052	51 <sup>2</sup>	5.5
	0.076	39	5.4
Dehydrocorticosterone	0.011	92	3.2
	0.034	65 <sup>2</sup>	5.4
	0.057	50	5.3
	0.083	36	5.4

Control experiments in which no steroid was used gave zero blank values. In two additional experiments it was found that when a known amount of corticosteroid was dissolved in 1 liter of water it was extracted essentially quantitatively by shaking the aqueous solution three times with 150 cc. lots of chloroform.

Table II gives information concerning the distribution of the various crystalline corticosteroids between benzene and water (Step 3). It is to be noted that the steroid was added to benzene which was extracted 10 times with an equal volume of water. The quantity of steroid remaining in the benzene and the quantity found in the combined water extract were deter-



mined. Column (e) of Table II shows that from 85 to 100 per cent of the steroid used was recovered in one or both of the two phases. Column (d) indicates that 17-OH corticosterone and 17-OH dehydrocorticosterone tend to pass quantitatively from benzene to water under the conditions of the experiment. On the other hand, only 38 per cent of the corticosterone and 24 per cent of the dehydrocorticosterone appeared in the water phase. Finally, desoxycorticosterone remained almost quantitatively in the benzene phase; only 2 per cent appeared in the water phase. Incidentally a sample of dehydroisoandrosterone similarly partitioned between benzene and water was found to remain essentially quantitatively in the benzene phase.

TABLE II

*Distribution of Various Crystalline Corticosteroids between Benzene and Water*

The steroid was added to 30 cc. of benzene, which was then extracted ten times with 30 cc. lots of water. The combined water extract was subsequently extracted four times with 45 cc. lots of chloroform. The respective corticosteroid contents of the benzene and the chloroform residues were then determined.

Steroid used	Amount of steroid added  (a)  mg.	Steroid recovered			
		Benzene phase  (b)  mg.	Water phase  (c)  mg.	$\frac{(c)}{(a)}$ (d)	$\frac{(b) + (c)}{(a)}$ (e)
None . . . . .		0.00	0.00		
17-OH corticosterone . . . . .	0.25	0.00	0.25	1.0	1.0
17-OH dehydrocorticosterone . . . . .	0.23	0.00	0.23	1.0	1.0
Corticosterone . . . . .	0.21	0.12	0.08	0.38	0.95
Dehydrocorticosterone . . . . .	0.21	0.15	0.05	0.24	0.95
Desoxycorticosterone . . . . .	0.48	0.40	0.01	0.02	0.85

The foregoing observations show that chloroform is a suitable solvent for extracting corticosteroids from aqueous solutions. The experiments with benzene show that the corticosteroids which lacked an oxygen on the 11th carbon atom were not easily extracted from benzene by water. They also suggest that 11-oxycorticosteroids with a hydroxyl group on the 17th carbon atom are extracted from benzene much more readily than 11-oxycorticosteroids without a hydroxyl group at C-17.

*Recovery of Crystalline Corticosteroids after Alkaline Washing of Chloroform Solution*—When the various crystalline corticosteroids are dissolved in chloroform, the chloroform solution may be washed with 0.1 N aqueous sodium hydroxide solution and by water according to the analytic procedure (Step 2) without significant losses of the steroid (maximum loss, 4 per cent).

*Recovery of Crystalline Corticosteroids Added to Urine*—The following ex-

periments were designed to test the efficiency of Steps 1 to 3 of the analytic procedure. For these experiments pools of fresh urine from normal adult human subjects were collected. The urine pools were separated into three or four equal aliquots. To two of these aliquots, nothing was added (control aliquots); to the others a measured amount of a crystalline corticosteroid was added prior to extraction. The various aliquots were then treated according to Steps 1, 2, and 3 of the analytic procedure and the "corticosteroid" content of the crude fractions thus obtained was determined by the colorimetric assay procedure.

The analytic data from six series of such experiments are given in Table III. The theoretical values (column (b)) for the urinary "corticosteroids" represent the sum of the average determined value for the two control aliquots (column (a)) and the mg. of crystalline corticosteroid added to the third or fourth aliquot, respectively. Column (c) gives the ratio of the determined to the theoretical values. Column (d) presents an estimation of the per cent of added corticosteroid actually recovered. Thus in Experiment 1 the average control value was 0.46 mg. per 1000 cc. of urine. In Experiment 1c, 0.73 mg. of 17-OH corticosterone was added. However, 1.28 mg. were recovered (column (a)). If it is assumed that 0.46 of the 1.28 mg. recovered was "corticosteroid" present in the urine aliquot prior to the addition of 17-OH corticosterone, then  $1.28 - 0.46$  or 0.82 mg. of 17-OH corticosterone was recovered. Since only 0.73 mg. of this steroid had been added, the estimated recovery was  $0.83/0.73 \times 100$  or 113 per cent (column (d)).

On the average the individual control values of Table III deviated from their respective mean values by  $\pm 10$  per cent (range  $\pm 0$  per cent (Experiment 6) to  $\pm 28$  per cent (Experiment 4)). In subsequent duplicate analyses, the values obtained have agreed within less than  $\pm 10$  per cent. Experiments 1 to 4 show that 17-OH corticosterone and 17-OH dehydrocorticosterone were recovered essentially quantitatively (columns (c) and (d)). On the other hand, the average recovery of corticosterone was 55 per cent and of dehydrocorticosterone 38 per cent (column (d)). These observations are in keeping with those of Table II which suggested that corticosteroids like corticosterone and dehydrocorticosterone, which lack a hydroxyl group at the 17th carbon atom, would not be recovered quantitatively by the analytic procedure.

Because these observations suggested that urine contained substances similar to the crystalline corticosteroids, a preliminary series of measurements of the "corticosteroid" content of the "crude fraction" of urines from normal and abnormal subjects was carried out. Though the results obtained were in the main consistent with biologic assay values reported by others, it was found that the colorimetric assay values obtained for patients

with Addison's disease having a tendency to hypoglycemia were not consistently lower than the values for normal subjects. This observation suggested that colorimetric assays carried out on the crude fractions might be

TABLE III  
*Recovery of Various Crystalline Corticosteroids Added to Urine*  
Extracts not purified by treatment with Girard's reagent.

Experiment No.	Urine volume	Steroid added*	Steroid recovered		(a) (b)	Estimated recovery of steroid added (d)
			Determined† (a)	Theoretical (b)	(c)	
	cc.	mg.	mg.	mg.		per cent
1a	1000	None	0.47			
b	1000	"	0.44 (0.46)			
c	1000	0.73 17-OH C	1.28	1.19	1.08	113
2a	500	None	0.21			
b	500	"	0.24 (0.23)			
c	500	0.54 17-OH C	0.78	0.77	1.01	104
3a	500	None	0.26			
b	500	"	0.24 (0.25)			
c	500	0.55 17-OH D	0.96	0.80	1.2	129
4a	500	None	0.51			
b	500	"	0.28 (0.39)			
c	500	0.55 17-OH D	0.78	0.94	0.83	71
d	500	0.54 C	0.66	0.93	0.50	50
5a	500	None	0.24			
b	500	"	0.33 (0.28)			
c	500	0.54 C	0.60	0.82	0.73	59
d	500	0.46 D	0.40	0.74	0.54	52
6a	1000	None	0.53			
b	1000	"	0.53 (0.53)			
c	1000	0.55 D	0.81	1.08	0.75	51

\* 17-OH C = 17-hydroxycorticosterone; 17-OH D = 17-hydroxydehydrocorticosterone; C = corticosterone; and D = dehydrocorticosterone.

† The figures in parentheses represent the average.

subject to errors of overestimation due to the presence of non-ketonic reducing agents. Accordingly, experiments were carried out to determine whether the crude fractions of urinary extracts could be further purified by treatment with Girard's Reagent T. This reaction has been used success-

fully for the separation of other ketonic urinary steroids from non-ketonic contaminants (15, 16).

*Recovery of Crystalline Corticosteroids in Pure Solutions in Ketonic Fraction after Treatment with Girard's Reagent T*—In nine experiments between 81 and 94 per cent (average 91 per cent) of the four respective crystalline 11-oxy corticosteroids was recovered in the ketonic fraction after treatment with Girard's Reagent T (Step 4 of the analytic procedure).

*Recovery of Crystalline Corticosteroids Added to Urine Extracts Prior to Treatment with Girard's Reagent T*—Four similar experiments on the recovery of crystalline corticosteroids added to an aliquot of the crude fraction of a urine extract prior to treatment with Girard's Reagent T were carried out. The corticosteroid content of the ketonic fraction of an equal aliquot to which nothing had been added was also measured. Accordingly, as in Table III, the theoretical values represent the sum of the control value and the mg. of crystalline corticosteroid added. (On the average 88 per cent of the crystalline corticosteroids added to the crude fractions was recovered in the ketonic fraction (range 81 to 94 per cent). Accordingly, it appears that Step 4 of the analytic procedure may be used without causing serious losses of corticosteroids. Experience has shown that assay values obtained on such ketonic fractions are often considerably lower than those obtained on the respective crude fractions. Most of the reducing material of the crude extract which does not appear in the ketonic fraction appears in the non-ketonic fraction.

Unfortunately, because of a lack of crystalline corticosteroids, it was not possible to test the efficiency of Steps 1 to 4 of the analytic procedure by recovery experiments similar to those of Table III. However, the data available indicate that the entire analytic procedure should permit recovery of approximately 90 per cent of the 17-OH corticosterone and 17-OH dehydrocorticosterone, 45 per cent of the corticosterone, 35 per cent of the dehydrocorticosterone, and 0 per cent of the desoxycorticosterone present in free (unconjugated) form in urine. That substances like desoxycorticosterone which lack an oxygen at the 11th carbon atom will probably not be recovered is of interest in view of the clinical observations discussed below. Also of interest is the fact that all the biologically active "cortin-like" material present in the urine extracts has been recovered essentially quantitatively after use of a purification procedure very similar to that employed here (9).

*Excretion of Corticosteroids by Normal and Abnormal Subjects*—Table IV gives preliminary measurements obtained with the analytic procedure described above. The daily output by three normal men and one normal woman was measured over 8 to 10 day periods. 89 per cent of the thirty-seven values for these subjects ranged between 0.12 and 0.34 mg. per day

(average value, 0.24 mg. per day). Analysis of these measurements showed that the chances were 2:1 that the value for a single day would fall within  $\pm 30$  per cent of the average value for that individual. There was essentially no difference between the values for the men and for the women. These observations, taken together with five additional measurements on five other normal adults indicate that values of 0.38 and higher should be considered abnormally high and values of 0.10 and below, abnormally low.

The remainder of Table IV gives measurements on patients with various conditions. It is seen that on the average results on patients with Addison's disease, hypothyroidism, or hypopituitarism tended to be low. Of the patients with Addison's disease or with hypopituitarism, only those with a definite tendency to hypoglycemia excreted 0.10 mg. or less of corti-

TABLE IV  
*Excretion of Corticosteroids by Normal and by Abnormal Subjects*

The results are expressed as mg. per 24 hours.

Condition	No. of subjects	No. of determinations	Urinary corticosteroids	
			Average	Range
1. Normal adults . . . . .	9	42	0.24	0.10-0.38
2. Addison's disease . . . . .	6	6	0.12	0.02-0.26
3. Hypothyroidism . . . . .	3	4	0.09	0.06-0.13
4. Hypopituitarism . . . . .	3	3	0.14	0.10-0.17
5. Cushing's syndrome . . . . .	3	4	4.20	0.90-12.0
6. Adrenal cortical virilism . . . . .	2	3	0.43	0.15-0.57
7. Simple hirsutism . . . . .	2	2	0.28	0.23-0.32
8. Burn and postoperative patients . . .	4	9	0.93	0.34-1.70

costeroids per day. On the other hand, values which, on the average, were abnormally elevated were obtained on patients with active Cushing's syndrome, adrenal cortical virilism, and on patients who had recently been severely burned<sup>5</sup> or operated upon. Two patients with simple hirsutism excreted normal amounts of corticosteroids.

#### *Comments*

The experimental data dealing with the analytic procedure with a few minor exceptions substantiate the observations of Venning *et al.* (9). The colorimetric measurement used here depends upon the fact that corticosteroids are reducing agents similar to sugars and are therefore susceptible of

<sup>5</sup> Assays carried out on the crude fraction of urine extracts of five other severely burned patients also gave values which were markedly elevated above assay values for normal crude fractions.

determination by suitable copper reagents. Since the urine contains a wide variety of reducing agents, the specificity of the analytic procedure depends upon the efficiency of the extraction and purification procedures. Assays made on extracts of water to which several gm. of glucose had been added gave zero values. The alkali washing of the chloroform solution (Step 2) should remove acidic substances. The information available suggests that only corticosteroids with an oxygen on the 11th carbon atom will tend to pass readily from benzene to water under the conditions used here (Step 3)<sup>6</sup> (17). Finally, it is probable that only ketonic reducing agents soluble in chloroform will appear in the ketonic fraction after treatment with Girard's Reagent T (Step 4). In these respects it is of interest that Venning *et al.* found the biologic activity per mg. of ketonic fraction residue<sup>7</sup> to approach closely that of crystalline 11-oxycorticosteroids (9). On the other hand, it would not be surprising to learn that urine contains biologically inactive "corticosteroids" as it contains biologically inactive as well as active 17-keto steroids (18). Should this be so, colorimetric assay values will not necessarily correspond exactly to biologic assay values.

The data obtained thus far for normal and abnormal subjects are in accord with the idea that the urinary substances measured were probably derived from the adrenal cortex. Furthermore, the fact that the lowest values occurred only in patients with a tendency to hypoglycemia suggests that the substances measured here may be an index of the rate of production of those adrenal cortical hormones which influence protein and carbohydrate metabolism rather than those which affect water and electrolyte metabolism. On the other hand, it is appreciated that the division of patients with Addison's disease into a group with and a group without hypoglycemia may be somewhat artificial. Accordingly, the relatively normal values reported here for some patients with Addison's disease raise the possibility that certain urine extracts may contain small amounts of reducing substances other than 11-oxycorticosteroids.

The detailed clinical aspects of these measurements will be reported elsewhere.

#### SUMMARY

A procedure for the extraction, purification, and colorimetric assay of urinary substances similar to 11-oxycorticosteroids has been described.

<sup>6</sup> There is a possibility that certain polyhydroxy steroids which lack an oxygen at C-11, but have a ketol side chain at C-17, may pass from benzene to water under the conditions of Step 3. This is suggested by an observation on a sample of crystalline  $\Delta^4$ -pregnene-3-one-17,20,21-triol which, when treated according to Step 3, was recovered almost quantitatively in the water phase.

<sup>7</sup> Prepared essentially according to the present analytic procedure.

Only 11-oxycorticosteroids with a 17-hydroxyl group are recovered essentially quantitatively by this procedure.

Preliminary observations on normal and abnormal subjects suggest that the substances measured are an index of the rate of secretion of those adrenal cortical hormones which influence protein and carbohydrate metabolism.

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# ESSENTIAL GROUPS OF CRYSTALLINE CHYMOTRYPSIN

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It is now generally recognized that, even in enzymes which do not require a "prosthetic group" for activity, certain parts of the molecule are much more concerned than others with the specific activity of the enzyme. These parts of the protein molecules may be thought of as "essential groups" which must be maintained intact in order for the enzyme to function. Attention has been focused especially upon the rôle of sulfhydryl groups, and it has been shown that there exists a large group of enzymes which are activated by reducing agents, which convert disulfide linkages to sulfhydryl groups, and are inactivated by reagents which oxidize these groups or combine with them. Included in this category are urease (1, 2), cathepsin, papain, and other plant proteases (3, 4),<sup>\*</sup> carbonic anhydrase (5), succinic dehydrogenase (6), triose phosphate dehydrogenase (7), glycerol dehydrogenase (8), pyruvate oxidase, and the enzyme for pyruvate condensation, carboxylase, ketoglutarate oxidase, malate oxidase, adenosinetriphosphatase (9), pancreatic lipase, certain fatty acid oxidases, certain amino acid oxidases, transaminase, monoamine oxidase (10), and amylase (11). On the other hand, it has become equally apparent that in a very large class of enzymes sulfhydryl groups are not required for activity. Enzymes of this type which have been studied are invertase (12), acid and alkaline phosphatase (13, 9), lactate oxidase, isocitrate oxidase, carbonic anhydrase,<sup>1</sup> polyphenol oxidase, catalase, uricase, cytochrome oxidase, flavoproteins (9), diamine oxidase, pepsin, trypsin (10), and pancreatic amylase (14).

The importance of tyrosine in the pepsin molecule has been demonstrated by the work of Herriott and Northrop (15, 16) and others (17), who have shown a decrease in activity when an acetylated, iodinated, or nitroso derivative of the tyrosine in the pepsin molecule is formed. Similarly, Sizer (12, 13) has indicated that when the tyrosine groups of invertase and acid or alkaline phosphatase are oxidized the activity is destroyed. Weill and Caldwell (18) have found that the reaction of  $\text{HNO}_2$  with the tyrosine of  $\beta$ -amylase results in a loss in activity.

Primary amino groups appear to play no rôle in determining the activity of such enzymes as pepsin (15) and  $\beta$ -amylase (18), but on the other hand constitute essential groups of pancreatic amylase (14) and alkaline phosphatase (19). While most of the studies on the rôle of sulfhydryl groups

<sup>1</sup> The results are contrary to Kiese and Hastings (5).



are fairly convincing, it is only in the case of pepsin that the importance of amino and tyrosyl groups has been clearly demonstrated.

The present investigation was undertaken with the purpose of extending this information on essential groups of enzymes by an investigation of the rôle played by sulfhydryl, disulfide, amino, and tyrosyl groups in determining the activity of purified chymotrypsin.

#### EXPERIMENTAL

In all experiments crystalline chymotrypsin from the Plaut Research Laboratory<sup>2</sup> was employed and was used at a final concentration in the digest of 1 mg. per ml. Several different buffers were employed for holding the digest at pH 8.0. All digestions were carried out in a water bath at  $37^{\circ} \pm 0.05^{\circ}$ . Except for the experiments with gelatin, the determinations of the activity of chymotrypsin were made by measuring the rate at which a reprecipitated filament of purified collagen (20, 21) was digested by the enzyme solution. A glass bead was tied to the filament before it was immersed in the enzyme solution; the end-point of digestion was taken as the time required for the filament to break and the bead to fall. The digestion time was measured with an automatic recording apparatus (22).

*Experiments with Oxidants and Reductants*—In view of the fact that certain oxidants or reductants may have specific effects in addition to those related to their oxidizing or reducing properties (2, 12, 13), it is often necessary to investigate the action on enzymes of a variety of such compounds. The enzyme activity can then be related to the oxidation-reduction potential of the digest.

Preliminary experiments indicated that  $1 \times 10^{-3}$  M solutions of most oxidants and reductants were not toxic and this concentration or less was used in subsequent studies. The oxidant or reductant was added to the enzyme for 5 minutes at  $37^{\circ}$  before the collagen filament was suspended in the solution. Before digestion was begun the oxidation-reduction potential was measured with a Beckman pH- $E_h$  meter at room temperature. A typical experiment of the twelve which were performed is presented in Fig. 1, from which it appears that chymotrypsin activity is independent of the oxidation-reduction potential of the medium from  $-400$  to about  $+500$  millivolts. A summary of all the studies which have been made<sup>3</sup> leads to the conclusion that chymotrypsin is unaffected by strong and weak reductants and relatively unaffected by mild oxidizing agents. These results can be interpreted as indicating that sulfhydryl or disulfide groups are not essential for chymotryptic activity. Strong oxidizing agents responsible

<sup>2</sup> Lehn and Fink Products Corporation, Bloomfield, New Jersey. The crystalline preparation contains about 40 per cent  $MgSO_4$ .

<sup>3</sup> Other reductants used were  $Na_2S_2O_4$ ,  $Na_2S_2O_3$ ,  $Na_2S$ , and cysteine, all of which showed essentially no effect, and KCN, which appreciably inhibited chymotrypsin.

for oxidation-reduction potentials greater than +540 millivolts progressively inactivate the enzyme the higher the  $E_h$ . On standing in solutions of high  $E_h$ , the chymotrypsin activity falls rapidly, while standing for a few hours at low potentials results in only a relatively slow loss of activity. The data on inactivation of chymotrypsin by strong oxidants are very similar to those on phosphatase (13) in which the loss in activity seemed to be associated with the oxidation of tyrosine. Iodine inactivates chymotrypsin as well as pepsin, in which enzyme it has been shown that it is the

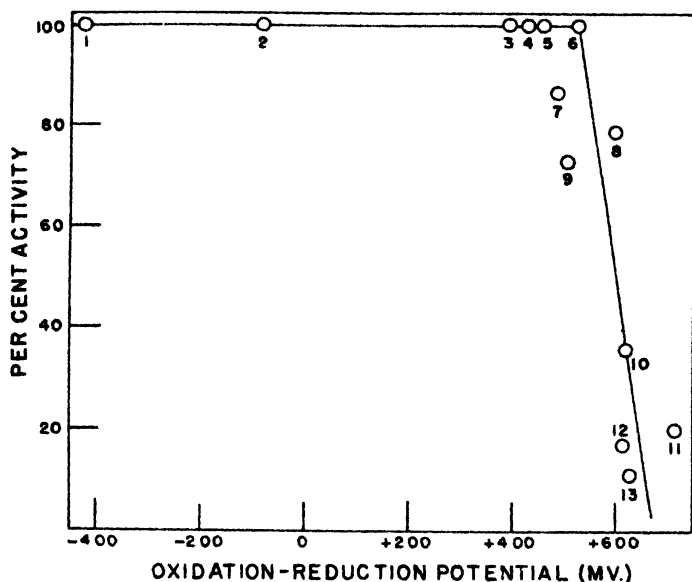


FIG. 1. Activity of chymotrypsin (as measured by the rate of digestion of a collagen filament) as related to the presence of oxidants or reductants in the digest. The solution at pH 7.9 contained 1 mg. of crystalline chymotrypsin per ml., 0.2 mg. of  $(\text{NH}_4)_2\text{HPO}_4$  per ml., and one of the following: 1,  $\text{H}_2$  activated with platinized asbestos; 2, 0.2 of saturated  $\text{H}_2\text{S}$ ; 3, control, nothing added; 4,  $5 \times 10^{-4} \text{ M}$   $\text{K}_4\text{Fe}(\text{CN})_6$ ; 5,  $2 \times 10^{-1} \text{ M}$  Br; 6,  $5 \times 10^{-1} \text{ M}$   $\text{K}_3\text{Fe}(\text{CN})_6$ ; 7,  $5 \times 10^{-1} \text{ M}$  iodoacetate; 8,  $5 \times 10^{-4} \text{ M}$   $\text{K}_2\text{Cr}_2\text{O}_7$ ; 9,  $2 \times 10^{-1} \text{ M}$  I-KI; 10,  $5 \times 10^{-1} \text{ M}$  I-KI; 11,  $5 \times 10^{-4} \text{ M}$   $\text{KMnO}_4$ ; 12,  $2 \times 10^{-4} \text{ M}$   $\text{KMnO}_4$ ; 13,  $5 \times 10^{-4} \text{ M}$  Br.

tyrosine group which is iodinated (16). Thus it appears likely that for chymotrypsin as well as for phosphatase and pepsin, tyrosyl groups are essential for activity.

The inactivation by strong oxidants of chymotrypsin, like phosphatase, is partially reversible. A 3-fold increase in activity can often be demonstrated by the addition to the partially inactivated enzyme of a reductant such as activated hydrogen or  $\text{K}_4\text{Fe}(\text{CN})_6$ . If inactivation has proceeded too far, however, it becomes irreversible. The reactivation of inactive

chymotrypsin seems analogous to the reactivation of acetylated pepsin by deacetylating the tyrosine groups of the enzyme (15).

Chymotryptic activity was also measured by the change in viscosity of 2 per cent gelatin at pH 7.5. This study demonstrated the fact that the action of oxidants and reductants is independent of the method used to measure enzyme activity.

*Acetylation with Ketene*—Ketene brings about the acetylation of primary amino, sulfhydryl, and phenolic hydroxyl groups of proteins, but does not react appreciably with the guanidino or aliphatic hydroxyl groups of amino acids and proteins (23, 24). The reaction with primary amines is much more rapid than with tyrosine (25, 26).

Freshly distilled ketene was prepared according to the method of Herriott and Northrop (15) and bubbled into the chymotrypsin solution at 0° at a rate of approximately one bubble per second. Considerable difficulty was encountered in controlling the pH of the solution, which even though highly buffered became acid, due to the conversion of ketene to acetic acid. The problem was solved by dissolving the enzyme in a 10 per cent suspension of  $\text{NaHCO}_3$ . The pH of this solution remained at 7.9 for about 1 hour and then gradually decreased. At successive intervals samples were taken from the solution being acetylated and the chymotrypsin activity measured in the usual way at 37°, with a collagen filament as substrate. Results of a typical experiment are presented in Fig. 2, from which it may be seen that acetylation of chymotrypsin by ketene is without effect for the first 30 minutes, after which the activity decreases quite rapidly, becoming zero in about 90 minutes. These results are in marked contrast with those reported by Little and Caldwell (14) for pancreatic amylase, who found that only 15 minutes acetylation caused a loss of 78 per cent of the activity, but a loss of only 13 per cent of the tyrosine groups. Gould (19) found an equally rapid loss in 10 minutes of the activity of phosphatase and concluded that primary amino groups were essential for activity in phosphatase as well as pancreatic amylase. The results on chymotrypsin acetylation are very similar to those on pepsin (15) and  $\beta$ -amylase (18), for which it has been shown that activity was lost not on acetylating the amino groups but only when the tyrosine groups were subsequently acetylated. Attempts to reactivate acetylated chymotrypsin by adjustment to pH 11 were unsuccessful. Reactivation by this procedure was accomplished with acetylated pepsin (15), but not with  $\beta$ -amylase (18).

*Experiments with Nitrous Acid*—It was hoped that further information on the rôle of essential groups in chymotrypsin could be obtained by treatment with  $\text{HNO}_2$ , since it is known that it oxidizes such groups as sulfhydryl, deaminates aliphatic amino groups of amino acids, and diazotizes phenolic hydroxyl groups (14, 17, 18). The deamination can be distin-

guished from the diazotization because the former proceeds very much more rapidly and in the presence of excess nitrite is of the second order, while the reaction with tyrosine is of the first order (14, 17).

The treatment of chymotrypsin with 1 M  $\text{HNO}_2$  was carried out at the recommended acidity of pH 4.6 and at  $0^\circ$ . To the solution were added 5 mg. per ml. of chymotrypsin and at successive intervals 0.5 ml. samples were removed and added to 2 ml. of phosphate buffer (final pH 7.7), and the enzyme activity measured as usual on collagen. A control at pH 4.6 with-

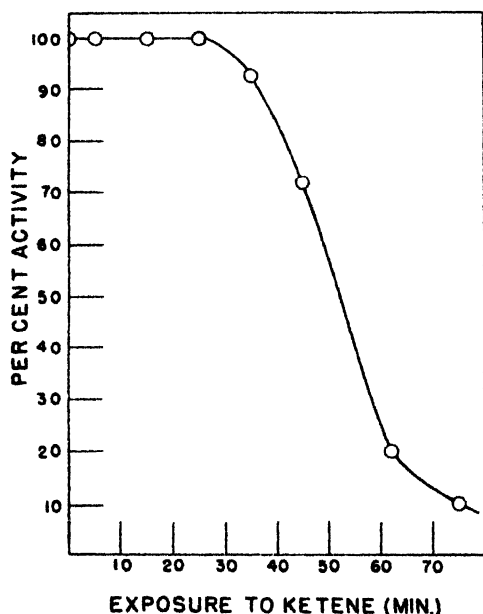


FIG. 2. Inactivation of crystalline chymotrypsin as a function of the time that ketene is bubbled through the solution. The activity of the enzyme was determined by the rate of digestion of a collagen filament.

out added  $\text{HNO}_2$  was run simultaneously to determine whether or not on standing at this acidity the enzyme was inactivated. The control showed no inactivation on standing for 22 hours.

Typical results are presented in Fig. 3, from which it appears that compared with pancreatic amylase and phosphatase (14, 19) chymotrypsin is inactivated very much more slowly and at a rate comparable with pepsin and  $\beta$ -amylase (17, 18). An analysis of the kinetics of inactivation (Fig. 3) shows that the diazotization of chymotrypsin is first order, indicating reaction of the  $\text{HNO}_2$  with tyrosine, since it would be second order under these conditions if the inactivation were due to the removal of amino groups by

$\text{HNO}_2$ . The reaction of the  $\text{HNO}_2$  with the tyrosine of chymotrypsin is also indicated by the gradual production of a yellow color in the solution due to the formation of the diazo compound. It should be pointed out that the formation of a yellow color might reflect the action of nitrous acid upon the tyrosyl nucleus rather than the formation of a diazo compound. Little and Caldwell (14) reported the progressive formation of a similar yellow diazo compound, as measured colorimetrically, during the later stages of the reaction between  $\text{HNO}_2$  and pancreatic amylase. The possi-

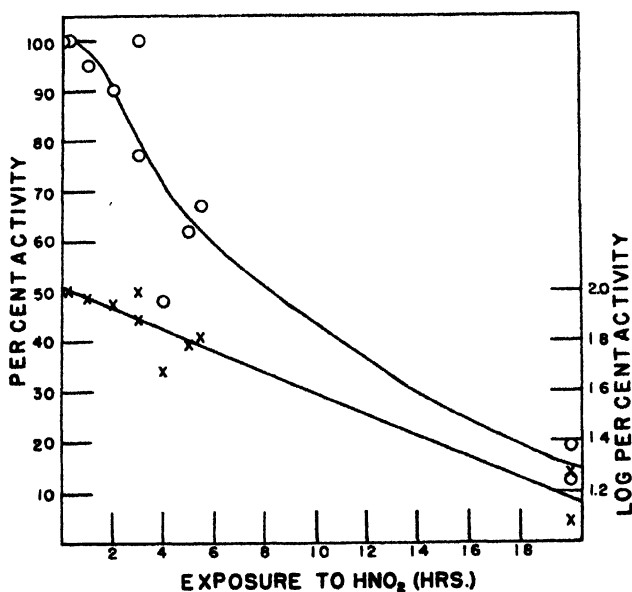


FIG. 3. Inactivation of chymotrypsin by 1 M  $\text{HNO}_2$  at pH 4.6 and  $0^\circ$ . The enzyme activity was determined by the rate of digestion of a collagen filament. The linear relationship in the logarithmic plot indicates that inactivation follows the course of a first order reaction.

bility that the loss of activity of chymotrypsin in  $\text{HNO}_2$  is due to the oxidation of sulfhydryl groups is excluded by the fact that partially inactivated enzyme cannot be reactivated by bubbling  $\text{H}_2\text{S}$  through the solution (see (18)). From these studies with  $\text{HNO}_2$  it may be concluded that neither amino nor sulfhydryl groups are required for chymotryptic activity but that tyrosine constitutes an essential group.

*Treatment with Phenyl Isocyanate*—Phenyl isocyanate forms phenylureido derivatives with a large number of different proteins. Hopkins and Wormall (27) believe the reaction to involve only the free amino groups of the lysine components and that further damage to the protein does not

occur, when the reaction is carried out at pH 8.0 and 0° with a ratio of protein to phenyl isocyanate of 2:1.

Duplicate solutions of chymotrypsin (1 mg. per ml.) were prepared in phosphate buffer at pH 7.6 at 0°. One was kept as the control, while to the other was added 0.5 mg. of phenyl isocyanate for each ml. of solution. The solution was shaken frequently during the reaction. The phenyl isocyanate solution turned cloudy and a precipitate of diphenylurea settled out. Samples were taken after 1 or more hours and the chymotrypsin activity measured in the usual way with a collagen filament as substrate. In four different experiments the activity of the isocyanate-treated enzyme varied from 92 to 99 per cent of the untreated control. These results indicate that phenyl isocyanate has relatively little effect in inactivating chymotrypsin. These data are interpreted as indicating that primary amino groups are not essential for chymotryptic activity, since in pancreatic amylase and phosphatase, in which amino groups are essential, phenyl isocyanate produced a rapid and practically complete inactivation (14, 19). At high concentrations phenyl isocyanate apparently brings about more drastic changes in the enzyme for, when used at 5 times the concentration of chymotrypsin (instead of 0.5), a rapid inactivation was produced.

*Experiments with Formaldehyde* Formaldehyde reacts readily at low concentrations with amino groups of proteins and at higher concentrations or longer periods of time it has a characteristic hardening or tanning action (28). It was felt that treatment of chymotrypsin with formaldehyde might yield further information concerning the relationship of amino groups to the activity of chymotrypsin.

To a solution of 1 per cent formaldehyde in phosphate buffer, pH 7.7 (room temperature) chymotrypsin was added to a final concentration of 1 mg. per ml. A control solution was similarly prepared but without the formaldehyde. Samples were drawn after 2 hours and tested for enzyme activity with the collagen filament technique. The solution containing the formaldehyde showed no enzyme activity, but this was shown to be due to the tanning of the collagen filament by the formaldehyde rather than the inactivation of chymotrypsin. In a second experiment the chymotrypsin was equilibrated with the formaldehyde for 2.5 hours. Both the control and experimental solutions were then dialyzed for 1.5 hours against a turbulent stream of tap water to free the experimental solution of excess formaldehyde. In one experiment the formaldehyde-treated enzyme had 92 per cent and in two experiments it had 100 per cent of the activity of the controls. These results indicate little if any inactivation by 1 per cent formaldehyde, and show that free amino groups are not essential for chymotryptic activity. With pancreatic amylase (18) and phosphatase (19), however, in which free amino groups are essential for activity, 1 per cent formaldehyde produced very extensive inactivation.

## SUMMARY

The activity of crystalline chymotrypsin has been studied as a function of the oxidation-reduction potential of the digest with a wide variety of compounds to poise the potential. It was found that the enzyme activity is independent of  $E_h$  from  $-400$  to  $+500$  millivolts, but that above  $500$  millivolts the enzyme is rapidly inactivated by strong oxidizing agents.

A detailed study has also been made of the effects of ketene, nitrous acid, phenyl isocyanate, and formaldehyde upon chymotryptic activity. Results with these compounds as well as those with oxidants and reductants are consistent in showing that primary amino, sulfhydryl, or disulfide groups are not required for chymotryptic activity, while tyrosine constitutes an essential group. Chymotrypsin is compared with other enzymes with reference to the importance of these groups in determining the activity of the enzyme molecule.

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## THE VERATRINE ALKALOIDS

### XXV. THE ALKALOIDS OF VERATRUM VIRIDE

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A review of the literature on earlier investigations of the alkaloid content of American hellebore or *Veratrum viride* Aiton has been given by Seiferle, Johns, and Richardson (1). In the earlier work the occurrence of only jervine, pseudojervine, and rubijervine had been described. In their study they were able to isolate, in addition to these alkaloids, germin and its ester, protoveratridine. Our own more recent studies of the alkaloids of this plant have now added to the list and have also necessitated reinterpretations of earlier data. In a previous paper (2) we have reported on one phase of this study concerned with the isolation of pseudojervine and the new alkaloid veratrosine, which were characterized as glucosides of jervine and veratramine respectively. These substances were found in the alcoholic extract of the plant material which followed preliminary extraction of the latter with benzene. A study of alkaloids contained in the benzene extract is the subject of the present report. The yield of partly crystalline crude basic material obtained after preliminary purification varied with different commercial samples of American hellebore, and any data given as to yields can be regarded only as approximations. About 11 gm. were extracted from each kilo of material. A preliminary separation of the bases was effected essentially as employed by Saito (3) in his study of *Veratrum grandiflorum* Loes. fil. By conversion to the sulfates a large sparingly soluble sulfate fraction was first obtained which after reconversion to the free bases was then transformed in alcoholic solution to the hydrochlorides. The sparingly soluble salt which crystallized was the hydrochloride of jervine, the principal alkaloidal constituent.

From the more soluble hydrochloride fraction a crystalline base was isolated which agreed very closely in properties with those recorded by Saito for veratramine. However, the analytical results obtained with our alkaloid were in closer agreement with the formulation  $C_{27}H_{39}O_2N$  than with that of  $C_{26}H_{35}O_2N$  derived by him. As a check on the identity of our substance, it was hydrogenated to a dihydro derivative with properties essentially as given by Saito for dihydroveratramine. Our analytical data again were more consistent with a formulation,  $C_{27}H_{41}O_2N$ . Saito's so called "diacetyl" derivative was also prepared. His analytical results as well as our own, however, are more satisfactory for a *triacetylveratramine*,



$C_{33}H_{45}O_5N$ . The latter on saponification with alkali yielded the substance described by him as a "low melting veratramine," but which we have since found to be the neutral *N*-acetylveratramine,  $C_{29}H_{41}O_3N$ .

Veratramine, therefore, is an unsaturated secondary base which possesses two acylatable hydroxyl groups. Since the presence of only one double bond was directly shown by hydrogenation, and since it is a secondary and

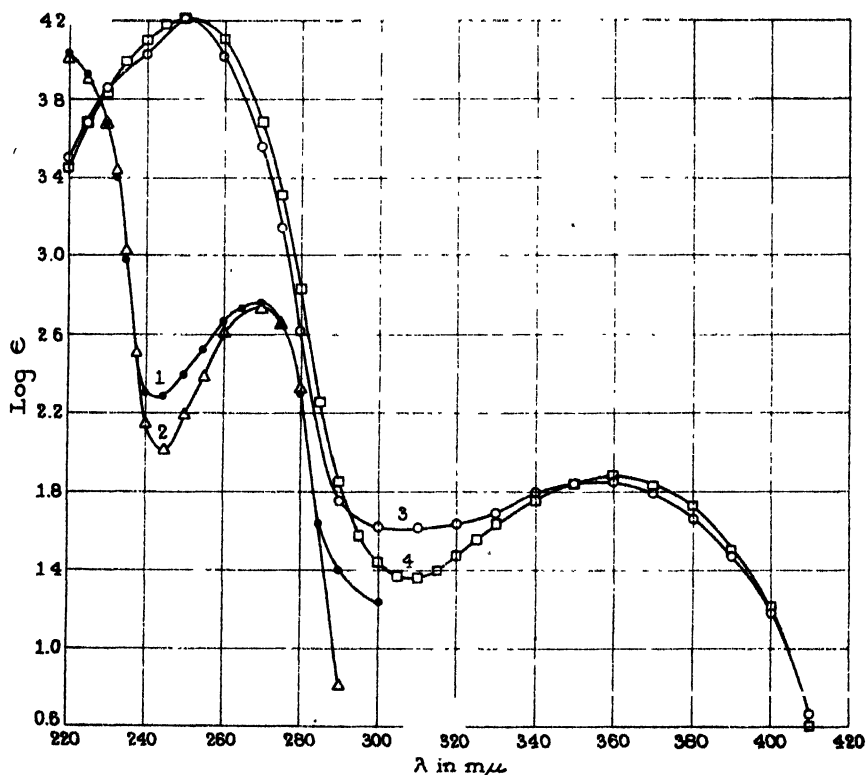


FIG. 1. Absorption spectrum curves. Curve 1, veratramine; Curve 2, dihydroveratramine; Curve 3, the new alkaloid; Curve 4, jervine.

presumably steroid base, there remained three points of unsaturation to be explained.

Information on this point was derived from the ultraviolet absorption spectrum of the alkaloid. Curve 1, Fig. 1, was obtained in ethanol solution with the Beckman quartz spectrophotometer. The similarity of the curve to that recorded in the literature for neoergosterol (4) and for trianhydrostrophanthidin (5), as contrasted to those recorded for simple conjugated double bonds, leaves little doubt that the three points of unsaturation are

contained in a benzene ring system in veratramine. If this is the case, and the alkaloid has the formula  $C_{27}H_{39}O_2N$ , then some modification of the regular sterol skeleton must be present, since in a regular sterol with 27 carbon atoms an angular methyl group would be inconsistent with the benzenoid character of the ring to which it is attached. In veratramine the heterocyclic portion could not give the ultraviolet absorption spectrum noted because of the secondary character of the nitrogen atom.

Since veratramine forms the glucoside, veratrosine, presumably analogous to pseudojervine, it is probable that there is present the usual hydroxyl group on carbon atom 3 and since no phenolic hydroxyl can be detected, Ring A should not be benzenoid. In work with jervine to be described on a later occasion, it will be shown that this alkaloid reacts in a way consistent with a hydroxyl group on carbon atom 3 and a  $\Delta^5$  double bond, since it can be converted through a ketone, presumably  $\Delta^4$ -jervone, to an allojervine which gives the color test of a 3-hydroxy- $\Delta^4$  derivative. In the case of veratramine, although it was changed readily to amorphous material with aluminum tertiary butylate, no evidence could be obtained that this was of the usual 3-oxo- $\Delta^4$  character. It appears probable, as a tentative interpretation, that Ring B rather than Ring C is the site of the benzenoid structure and that, in consequence, the usual steroid angular carbon atom 18 attached to carbon atom 10 has been shifted in veratramine to carbon atom 1, as has been assumed to occur in trianhydrostrophanthidin (6).

Although the veratrine alkaloids are unquestionably steroid, attempts to relate any one of them directly to the sterols by dehydrogenation to Diels' hydrocarbon have in each case given a different result. Rubijervine is precipitated by digitonin and gives the transformations of cholesterol (7) in regard to a 3( $\beta$ )-hydroxy- $\Delta^b$  structure, but no Diels' hydrocarbon was detected upon dehydrogenation. Instead, a lower melting isomeric hydrocarbon (8) was obtained which was shown to possess a phenanthrene ring system by ultraviolet absorption spectrum study. Its properties and those of its trinitrobenzene derivative approximated closely the values recorded in the literature for 1'-methyl-1,2-cyclopentenophenanthrene (9). The possibility must also be considered, among others, that this hydrocarbon can prove to be 5-methyl-1,2-cyclopentenophenanthrene. The formulation of the latter would be consistent with a methyl group on carbon atom 1. It is now our intention to determine the identity of this hydrocarbon by comparison with synthetic material. Although this substance is a high temperature product, if it should prove to be 1'-methyl-1,2-cyclopentenophenanthrene, its formation might raise the question as to whether carbon atom 15 in rubijervine is the site of a methyl group instead of carbon atom 13.

In the case of dihydroveratramine, the absorption curve, Curve 2, Fig. 1,

has been found to resemble quite closely that of veratramine, and therefore indicates not only retention of the benzenoid structure but that the double bond of veratramine which can be hydrogenated is not conjugated with the benzene ring. If the base contains the usual angular methyl group on carbon atom 13, this double bond would be restricted to Ring D or the sterol side chain which carries the nitrogen atom.

Contrary to jervine, veratramine was found to be relatively resistant to acid and was recovered unchanged when submitted to the action of methyl alcoholic HCl. As previously described, veratrosine yields veratramine and *d*-glucose on hydrolysis.

An appreciable fraction of alkaloidal material remained in solution as more soluble salts after precipitation of the above sparingly soluble sulfate fraction. A partial separation of the bases recovered from this fraction was effected by chromatographing through aluminum oxide. The major portion was gradually eluted from the column with benzene which contained 2.5 per cent of methanol. The crystalline substance which emerged with amorphous material in the early fractions proved to be identical with the isorubijervine first obtained from *Veratrum album*. This overlapped into later fractions which contained rubijervine. The separation of rubijervine from isorubijervine and a relatively large amount of amorphous material was effected by taking advantage of the sparing solubility in moist chloroform of rubijervine which contained water of crystallization.

After the elution of rubijervine, at first only amorphous material was obtained, but fractions were then encountered from which it was possible to isolate a new crystalline alkaloid in very small amount. 12 kilos of plant material yielded about 0.5 gm. This alkaloid contained solvent and, after preliminary softening, melted up to about 170–175°, then crystallized again and remelted at 272–274°. The analytical data suggested a provisional formula,  $C_{27}H_{41}O_4N$  (or  $C_{27}H_{39}O_4N$ ). This was supported by analyses of a neutral *N*-acetyl derivative,  $C_{29}H_{43}O_5N$ , and of a nitroso derivative,  $C_{27}H_{40}O_5N_2$ . The formation of these derivatives showed the secondary basic character of the alkaloid which is thus the third secondary alkaloidal base to be isolated in succession to jervine and veratramine (and their glucosides). The *N*-acetyl derivative was obtained by saponification of the uncrystallized product of the direct acetylation of the alkaloid. The number of OH groups involved in this intermediate acetyl derivative was not determined. The alkaloid did not react with hydroxylamine.

The ultraviolet absorption spectrum, Curve 3, Fig. 1, obtained with the alkaloid indicates the presence of conjugated double bonds and resembles the curve obtained on a previous occasion with jervine (10), except that a second but lower broad band with a maximum at 360  $m\mu$  has been found. Since the earlier observations with jervine had not been extended so far,

the latter has now been reexamined and has been found to exhibit a similar second broad band with a maximum at 360  $m\mu$ , Curve 4, Fig. 1. Although certain implications might be drawn from these observations, their interpretation will be left to a later occasion in connection with other data which will be available.

There was evidence of the presence of other crystalline alkaloids in fractions from which the new alkaloid was obtained but in amounts too small to make profitable their study at the moment. Further elution of the column with benzene which contained more methanol continued to yield fractions of amorphous material from which small amounts of germine were eventually obtained. Germine was more readily isolated, although in small amount, by continued extraction with chloroform of the aqueous phase which remained after the preliminary removal of the more easily extracted major alkaloid fraction discussed above. The identity of this germine was confirmed by the preparation of the acetonyl derivative (11).

A relatively large amorphous fraction of alkaloid material still remained which will require future study.

#### EXPERIMENTAL

Ground roots and rhizomes of commercial *Veratrum viride* were extracted essentially as previously described (2) in portions of 2 kilos with benzene and dilute ammonia. The combined benzene extracts from 6 kilos were concentrated under reduced pressure and brought to 4 liters in benzene. This was extracted repeatedly with portions of a total of 5 liters of 5 per cent aqueous acetic acid. The free bases were reprecipitated from the aqueous extract with excess 25 per cent NaOH and then reextracted with benzene. A small persistent precipitate which gradually separated at the interface and retarded separation was discarded and the benzene solution after washing with water was dried over sodium sulfate. The remaining alkaline aqueous phase by further repeated extractions with chloroform yielded the germine to be described below. The benzene solution on concentration to dryness gave a partly crystalline residue of approximately 65 gm. of a crude alkaloid mixture. The solution of this material in 950 cc. of 5 per cent acetic acid was treated with 135 cc. of saturated ammonium sulfate solution. The copious rather gelatinous precipitate was warmed, which caused partial formation of minute microcrystalline aggregates. It was collected with 5 per cent ammonium sulfate solution. The precipitate was resuspended in fresh wash solution and recentrifuged several times to insure proper washing of the crude sulfate. The soluble sulfate fraction contained in the mother liquors was worked up as given below. The sulfate was then resuspended in water and treated with excess NaOH solution. The mixture was shaken thoroughly with chloroform to redissolve the lib-

erated bases. The extract was separated by centrifugation and after washing with water was cleared with sodium sulfate and concentrated to small bulk. After addition of absolute ethanol, all residual chloroform was boiled off. To the mixture (250 cc.), which had partly crystallized, HCl (sp. gr. 1.19) was carefully added in slight excess. Immediate deposition of jervine hydrochloride occurred. After cooling and collection with absolute ethanol, 32 gm. were obtained. Partial concentration of the mother liquor under reduced pressure yielded a small additional fraction. If carried too far, this material was contaminated with the salt of veratramine.

When the hydrochloride was dissolved in hot, somewhat diluted ethanol and decomposed with ammonia, the base separated as needles which melted at 240–241° (uncorrected).

$$[\alpha]_D^{25} = -146^\circ \text{ (} c = 0.99 \text{ in absolute ethanol)}$$

$C_{27}H_{39}O_2N$ . Calculated, C 76.18, H 9.24; found, C 76.24, H 9.22

*Veratramine*—The mother liquor from crude jervine hydrochloride was treated with excess ammonia and carefully diluted to incipient turbidity. Veratramine gradually separated as a copious deposit which increased on further dilution. The alkaloid was collected with 50 per cent ethanol and additional amounts were obtained from the mother liquor. The yield was 7 to 8 gm.

On recrystallization from 95 per cent ethanol, it separated as delicate needles which melted at 204–207° after preliminary sintering and contained water of crystallization; the air-dried material showed no ethoxyl content.

$$[\alpha]_D^{25} = -69^\circ \text{ (} c = 0.98 \text{ for the anhydrous substance in methanol)}$$

For analysis, the substance was dried at 110° and 0.2 mm.

$C_{27}H_{39}O_2N$ . Calculated. C 79.16, H 9.60

Found. (a) " 78.88, " 9.57

(b) " 78.69, " 9.73

Saito reported a rotation of  $[\alpha]_D^{19} = -70^\circ$  ( $c = 1.065$  in methanol) apparently for the undried material.

Attempts to raise the melting point to 209.5–210.5° as reported by Saito were unsuccessful by simple recrystallization. This was accomplished, however, as follows, which suggests that small persistent amounts of contaminating jervine may have been removed in the process.

0.2 gm. of the product was dissolved with chilling in 4 cc. of methanol saturated at 0° with HCl. The solution was allowed to come to room temperature in a sealed tube. After 1.5 hours the base was recovered by dilution, addition of alkali, and extraction with chloroform. It crystallized from dilute ethanol as delicate needles and after recrystallization melted at

209–210.5°. There was no appreciable depression when mixed with veratramine.

$$[\alpha]_D^{25} = -68^\circ \text{ (} c = 0.88 \text{ for the anhydrous substance in methanol)}$$

For analysis, the substance was dried at 110° and 0.2 mm.

Found, C 79.12, H 9.65

As a check on its identity, it was acetylated and the product was found to be indistinguishable in properties and melting point from the triacetyl derivative described below.

*Dihydroveratramine*—0.2 gm. of veratramine was hydrogenated with 50 mg. of platinum oxide catalyst in acetic acid solution, as described by Saito. The base recovered from the reaction was obtained as leaflets from dilute ethanol which melted at 198–200° after slight preliminary sintering. Saito reported 197–198°.

For analysis, it was dried at 110° and 0.2 mm.

$C_{27}H_{44}O_2N$ . Calculated, C 78.77, H 10.05; found, C 78.75, H 10.10

*Triacetylveratramine*—This was prepared, essentially as described by Saito, by boiling in acetic anhydride but for only 30 minutes. The residue obtained on concentration crystallized directly from dilute ethanol. On recrystallization from 95 per cent ethanol, it formed needles which at about 120–130° showed a change in appearance, presumably due to loss of solvent, and then melted at 204–206° after preliminary sintering. Saito reported 205–206.5° for his “diacetylveratramine.”

For analysis, it was dried at 110° and 0.2 mm.

$C_{33}H_{46}O_4N$ . Calculated. C 73.97, H 8.47

Found. (a) “ 74.00, “ 8.25

(b) “ 74.09, “ 8.47

*Acetylveratramine*—80 mg. of the triacetyl derivative were refluxed for 2 hours in 2 cc. of 2 N NaOH in absolute ethanol. On dilution to incipient turbidity, it gradually crystallized. When recrystallized from dilute ethanol, it formed almost rectangular leaflets which melted at 177–180° after slight preliminary softening. Saito reported 179–180°. It did not dissolve in dilute acids.

For analysis it was dried at 110° and 0.2 mm.

$C_{31}H_{44}O_2N$ . Calculated, C 77.11, H 9.16; found, C 77.07, H 9.21

The combined mother liquor and washings of the crude insoluble sulfate fraction were made alkaline and repeatedly extracted with chloroform.

The washed extract was cleared with sodium sulfate and concentrated. A resinous residue of mixed bases was obtained which varied in different experiments from 16 to about 25 gm. for 6 kilos of plant material.

32.5 gm. of mixed bases were dissolved in 200 cc. of benzene and passed through a column of 650 gm. of active alumina. Attempted elution with twelve 100 cc. portions of benzene was ineffective and so 2.5 per cent of methanol in benzene was used. After a liter had passed through, dissolved material began to emerge from the column and was collected in 100 cc. portions. The amounts obtained in each fraction after removal of solvent are recorded in Table I. Data obtained beyond Fraction 32, how-

TABLE I  
*Chromatogram of Bases from Soluble Sulfate Fraction*

Fraction No.	Weight eluted	Fraction No.	Weight eluted
2.5% methanol		5% methanol	
	gm.		gm.
1	0.37	17	0.05
2	1.9	18	0.05
3	3.05	19	0.05
4	4.1	20	0.05
5	3.6	21	0.05
6	3.0	22	0.06
7	2.02	23	0.08
8	1.35	24	0.10
9	1.05	25	0.10
10	0.71	26	0.10
11	0.35	27	0.15
12	0.27	28	0.19
13	0.17	29	0.18
14	0.15	30	0.14
15	0.09	31	0.10
16	0.05	32	0.09

ever, in which 10 per cent methanol was employed, are not recorded. About 70 per cent of the original weight was recovered in Fractions 1 through 15.

Each fraction up to Fraction 16 was dissolved in a small volume of chloroform and treated with a few drops of water to furnish water of crystallization for rubijervine. The latter crystallized only on seeding in small amount in Fraction 5, then in gradually increasing relative amounts up to Fraction 15, and again diminished. In each case it was collected with moist chloroform. The combined yield was 1.5 gm. On recrystallization from 95 per cent ethanol, it crystallized as needles which melted at 242–244°.

$$[\alpha]_D^{20} = +20.5^\circ \text{ (} c = 0.97 \text{ in absolute ethanol).}$$

For analysis, it was dried at  $110^\circ$  and 0.2 mm.

$C_{27}H_{48}O_2N$ . Calculated, C 78.38, H 10.49; found, C 78.38, H 10.61

Fractions 2 to 5 (after filtration from rubijervine) were concentrated to remove chloroform, and the residue was dissolved in ethanol. On careful dilution, isorubijervine crystallized copiously as needles and was collected with 70 per cent ethanol. 2.3 gm. were obtained, which melted at  $216^\circ$ .

The rubijervine mother liquors of Fractions 6 to 15 were combined and, after removal of chloroform, yielded slowly an additional 1.2 gm. of crude isorubijervine.

On recrystallization from dilute ethanol, isorubijervine was obtained which contained solvent and melted at  $213\text{--}215^\circ$  after preliminary sintering.

For analysis, it was dried at  $110^\circ$  and 0.2 mm.

Found, C 78.42, H 10.57

In other cases, the relative and absolute yields of the two alkaloids varied considerably and appeared to depend somewhat upon striking optimum manipulative conditions for their separation from the larger amount of still uncharacterized alkaloid material.

*The Alkaloid  $C_{27}H_{41}O_4N$  (or  $C_{27}H_{39}O_4N$ )*.—As Fraction 16 emerged from the column, the eluting solvent was changed to 5 per cent methanol in benzene. Fractions 20 to 32 were found to contain a new alkaloid which crystallized from a methanol solution of the residues obtained after removal of the eluting solvent. Crystallization was induced by seeding with material obtained in preliminary work. The yield was 0.48 gm. In another experiment, 44 gm. of chromatographed alkaloid mixture (from 12 kilos of plant material) yielded 0.5 gm. of the new alkaloid.

On recrystallization from methanol, it formed six-sided platelets, some sufficiently long and narrow to constitute needles. After preliminary sintering above  $130^\circ$ , it gradually softened to a melt with effervescence at about  $170\text{--}175^\circ$ , crystallized again on further heating, and then melted at  $272\text{--}274^\circ$ . It dissolved readily in chloroform, acetone, appreciably in benzene, rather sparingly in methanol or ethanol, and very sparingly in ether.

$$[\alpha]_D^{20} = -78^\circ \text{ (} c = 0.63 \text{ in methanol)}$$

It contained solvent and for analysis was dried at  $110^\circ$  and 0.2 mm.

$C_{27}H_{41}O_4N$ .	Calculated.	C 73.08, H 9.32
$C_{27}H_{39}O_4N$ .	"	" 73.42, " 8.91
	Found. (a)	" 73.41, " 9.30
	(b)	" 73.10, " 9.42
	(c)	" 72.93, " 9.24



The new alkaloid did not yield a sparingly soluble sulfate. The hydrochloride crystallized in the presence of excess dilute HCl.

*The N-Acetyl Derivative*—The alkaloid was boiled for a minute with acetic anhydride. After decomposition with water, the mixture was directly extracted with chloroform. The latter after concentration left a resin of the polyacetyl derivative which did not crystallize. It was dissolved in a small volume of methanol, treated with excess alkali, and boiled a few minutes to saponify the ester groups. The diluted mixture was acidified with  $\text{H}_2\text{SO}_4$  and all neutral material was extracted with chloroform. The latter on concentration yielded a resin which finally crystallized from a small volume of dilute methanol. It formed flat micro prisms or short broad needles which contained solvent and melted at  $168\text{--}170^\circ$ .

For analysis, it was dried at  $100^\circ$  and 0.2 mm.

$\text{C}_{29}\text{H}_{45}\text{O}_5\text{N}$ . Calculated, C 71.70, H 8.93; found, C 71.41, H 9.07

*The Nitroso Derivative*—50 mg. of the alkaloid were dissolved in 2 cc. of water and 0.2 cc. of acetic acid. Upon addition of sodium nitrite solution, it soon became a mass of crystals. After standing, the mixture was extracted with chloroform and the extract was shaken with dilute  $\text{H}_2\text{SO}_4$ , then with water, and dried. This yielded on concentration material which crystallized from dilute ethanol as lustrous, microscopic, six-sided platelets, and melted at  $263\text{--}265^\circ$ .

It contained solvent and for analysis was dried at  $110^\circ$  and 0.2 mm.

$\text{C}_{27}\text{H}_{40}\text{O}_5\text{N}_2$ . Calculated. C 68.60, H 8.53  
Found. " 68.91, " 8.48

*Germine*—The above alkaline aqueous phase which remained after re-extraction of the crude alkaloid mixture with benzene was subsequently extracted twelve times with chloroform. The combined extracts after drying and concentration yielded a residue of 1.8 gm. When dissolved in methanol and seeded with germine, crystallization occurred. 0.23 gm. of alkaloid was obtained, although continued extraction of the aqueous phase yielded additional amounts of the alkaloid. On recrystallization from methanol, it formed small prisms which melted gradually at  $163\text{--}168^\circ$  to a paste filled with bubbles.

$[\alpha]_D^{25} = +5^\circ$  ( $c = 0.97$  in 95% ethanol)

For analysis, it was dried at  $110^\circ$  and 0.2 mm.

$\text{C}_{27}\text{H}_{44}\text{O}_5\text{N}$ . Calculated, C 63.61, H 8.51; found, C 63.52, H 8.70

For further characterization, the acetonyl derivative was prepared as previously described (11) for acetonylgermine through the hydrochloride,

which was then converted to the free base. The latter agreed in melting point and other properties with acetonylgermine.

$C_{27}H_{41}O_4N$ . Calculated, C 65.53, H 8.62; found, C 65.92, H 8.66

All analytical data reported here were obtained by Mr. D. Rigakos of this laboratory.

#### SUMMARY

A study of the alkaloids contained in the benzene extract of the roots and rhizomes of commercial *Veratrum viride* Aiton has resulted in the isolation of rubijervine, isorubijervine, jervine (the preponderating base), veratramine, germine, and a new alkaloid,  $C_{27}H_{41}O_4N$  (or  $C_{27}H_{39}O_4N$ ). The formulation of veratramine has been revised to  $C_{27}H_{39}O_2N$ . A triacetyl derivative is formed on acylation which by saponification yields an N-acetylveratramine. Absorption spectra data consistent with a benzenoid structure have been obtained with the alkaloid and its dihydro derivative. It must therefore possess four double bonds. In interpreting its structure, a rearrangement of one of the usual steroid angular methyl groups has been considered.

The new alkaloid,  $C_{27}H_{41}O_4N$ , is also a polyhydroxy secondary base which yields an N-acetyl derivative and a nitroso derivative but no oxime.

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# THE MINERAL COMPOSITION OF HUMAN EPIDERMIS\*

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Our investigations on epidermal methylcholanthrene carcinogenesis in mice have demonstrated the utility of certain methods of direct chemical analysis of epidermis (1, 2). It seemed desirable to apply these techniques to normal human epidermis with the idea of later investigating human squamous cell carcinomata and of comparing the chemical changes in epidermal carcinogenesis in human and mouse epidermis.

## EXPERIMENTAL

The epidermis was cleanly separated from the dermis by the procedure of Baumberger, Suntzeff, and Cowdry (3). Weighed samples of the epidermis (250 to 600 mg., wet weight) were completely ashed at 450° in a muffle furnace in silica crucibles, and the mineral content on the hydrochloric acid solution of the ash (one sample was used for the determination of one metal) was determined as follows: sodium polarographically as sodium uranyl zinc acetate (4); magnesium polarographically as magnesium hydroxyquinolate (5); potassium as potassium chloroplatinate by the procedure of Consolazio and Talbott (6); and calcium as calcium oxalate by the method (on a micro scale) of Lindner and Kirk (7). Due to the fact that a considerable area of skin is required to give sufficient epidermis for a single analysis, some of our samples were limited to a single determination of one of the metals. However, in cases of leg and arm amputations and of radical breast removals,<sup>1</sup> sufficient epidermis was made available for measurements of two to four of the metals (potassium, sodium, calcium, and magnesium) in duplicate. Skin removed from gangrenous legs was taken at a considerable distance from the lesion, as were also samples involving osteosarcomas. Breast skin was used only if it appeared normal and was not adjacent to areas of induration. In all cases the whole skin was removed as soon as possible after operation, and after the epidermis was washed with distilled water it was separated from the dermis. Specimens

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of all samples were fixed and stained for histological control to insure our dealing with normal epidermis. Prior to operation, some of the samples had been painted with metaphen, but a comparison of the analyses between painted and unpainted areas did not reveal any significant differences in metal content.

TABLE I  
*Mineral Composition of Human Epidermis*

Sample	Age and sex	Metal per 100 mg. epidermis			
		K	Na	Mg	Ca
		mg.	mg.	mg.	mg.
Leg, diabetic gangrene	72 M.	0.279	0.111	0.021	
" " "	79 "	0.284	0.115	0.018	
" " "	73 "	0.288	0.112	0.019	
" " "	62 "		0.132	0.021	
" " "	"	0.308	0.162	0.018	0.014
" " "	"	0.279	0.122		
" arteriosclerotic gangrene	67 "	0.275	0.104		0.019
" " "	74 "	0.304			0.016
" " "	59 "	0.278	0.140	0.018	0.014
Arm, osteogenic sarcoma	"	0.301	0.110	0.017	0.018
" " "	60 "	0.313	0.120	0.015	0.015
Leg, arteriosclerotic gangrene	49 "	0.341	0.111	0.021	
" diabetic gangrene	65 "	0.353	0.102	0.018	0.014
" " "	"	0.330	0.109		0.017
" " "	59 "	0.333			0.014
" " "	67 "	0.334	0.138		
" " "	64 F.	0.348	0.144	0.016	0.016
" " "	66 M.	0.357		0.018	
Leg, not gangrenous	19 "	0.374	0.137	0.017	0.015
Arm, osteogenic sarcoma	15 F.	0.377		0.018	
Breast carcinoma	52 "	0.345			0.015
" "	39 "	0.371	0.103	0.020	0.015
Average.....		0.322	0.122	0.018	0.015

### Results

In our preliminary work, in which there was only sufficient epidermis for a single analysis or for an analysis in duplicate, fourteen samples contained 0.263 to 0.277 mg. of potassium per 100 mg. of epidermis and six others had 0.304 to 0.340 mg. per 100 mg. In the same category sixteen samples of epidermis had 0.014 to 0.018 mg. of calcium per 100 mg. of epidermis. These samples for both metals were from amputations of legs showing diabetic gangrene, excised carcinomatous breasts, and one from a leg with osteosarcoma.

The results on the samples of skin from one area of the body which sufficed for the determination of two to four of the metals in duplicate are shown in Table I, and are expressed as mg. of metal per 100 mg. of epidermis. The values for the potassium content of these samples are tabulated in two groups; in one they varied from 0.275 to 0.313, in the other from 0.330 to 0.372 mg. per 100 mg. In the limited samples available there appeared to be no relationship between potassium content and the age or sex of the individual, the kind of associated lesion, or the location of the skin. The sodium contents were less uniform. The amounts of magnesium and calcium are in good agreement and the contents of calcium are about the same as those reported above in which there was only sufficient skin for the determination of one metal. The over-all average of each metal per 100 mg. of epidermis was as follows: 0.322 mg. of K, 0.122 mg. of Na, 0.018 mg. of Mg, and 0.015 mg. of Ca.

The technique used for separating the epithelial cells (epidermis) from the underlying dermis (3) has the advantage of giving pure cellular material for chemical analysis, a procedure which has made possible our studies on epidermal carcinogenesis in mice (2) and should be of obvious value for other investigations. The same method may well be employed to collect pure samples of other epithelia (buccal mucous membrane, nasal mucous membrane, etc.) for biochemical investigations.

#### SUMMARY

The mineral composition of human epidermis with respect to potassium, sodium, calcium, and magnesium was investigated. The average amount of each metal per 100 mg. of epidermis was 0.322 mg. of K, 0.122 mg. of Na, 0.018 mg. of Mg, and 0.015 mg. of Ca. With the limited material at our disposal no relationship between the mineral content and the age, sex, the associated lesion, or the location of the skin was apparent.

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# THE ESTIMATION OF FREE FORMALDEHYDE BY DIFFUSION\*

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The procedure to be described was devised for the purpose of determining the amount of free or uncombined formaldehyde in bacterial toxins during the process of rendering them non-toxic. The determination of formaldehyde is made without changing the pH and without diluting the sample to be tested. Disturbance of amino acid-formaldehyde equilibria existing in solution is therefore minimum.

The following principle is employed in the diffusion procedure. Formaldehyde vapor is allowed to diffuse from the surface of a solution to be analyzed into a membrane containing phenylhydrazine hydrochloride. A red color is developed in the membrane by adding ferrieyanide and acid and compared with colors developed at the same time in a similar manner from standard solutions of formaldehyde.

The outstanding merit of the phenylhydrazine-ferrieyanide reaction for formaldehyde as applied in this procedure is its remarkable sensitivity. Notwithstanding the very low volatility of formaldehyde in dilute solutions, samples containing 2 to 1000  $\gamma$  of formaldehyde per cc. are suitable for analysis. Presumably only a small proportion of the formaldehyde in or near the surface of the solution takes part in the reaction.

Rimini (1) evidently first used phenylhydrazine in a colorimetric test for formaldehyde. As modified by Schryver (2) and others (3, 4), the reagents have been used to test for free formaldehyde. In these methods the reagents (one of which was fairly concentrated HCl or NaOH) were added to the solution to be analyzed. The color that was developed depended, in part, on the amount of formaldehyde which remained dissociated from combinations with amino acids after the addition of the reagents.

The procedure and apparatus are designed so that the formaldehyde can be determined to  $\pm 10 \gamma$  per cc. in solutions containing less than 200  $\gamma$  per cc. and to  $\pm 5$  per cent for higher concentrations.<sup>1</sup> The procedure is performed with apparatus which is readily constructed and a technique

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Cincinnati.

<sup>1</sup> From 0 to 20  $\gamma$  results ( $\pm 2 \gamma$ ) can be obtained with a cellulose nitrate film. With the Visking membrane, concentrations higher than 1000  $\gamma$  per cc. can be determined with slight sacrifice in accuracy.



which is easily mastered. Significant changes in formaldehyde concentrations during the detoxication process are easily and quickly determined on small samples of toxin-toxoid.

The method has been applied to the investigation of combinations of formaldehyde with amino acids over a wide range of pH. It has been found that the rate at which formaldehyde diffuses from pure dilute solutions is dependent on the pH of the solution. These variations with pH are recorded and discussed.

#### EXPERIMENTAL

##### *Apparatus—*

*Test block* (Fig. 1). Twenty holes, 0.25 inch apart, are drilled with a 0.75 inch drill in two rows equidistant from the center line on a block of resin (such as Lucite, Plexiglas, or Formica) measuring  $2.25 \times 1 \times 12$  inches. The large diameter of the drill is allowed to penetrate the block 0.3 of an inch. A block drilled to a depth of 0.5 inch is satisfactory and may be preferable when the concentrations of formaldehyde to be determined are 500 to 1000  $\gamma$  per cc. (Holes drilled to a depth of 1 inch have not been satisfactory.)

The block is now paraffined. The holes are filled with molten paraffin and the excess paraffin is removed by vigorously shaking the block upside down. Lucite was selected from the currently available resins because the surfaces of the blocks were plane. Formica blocks have the same advantage.<sup>2</sup> A drilled Formica block should be heated to 100° until it gives a negative test for formaldehyde when exposed to the membrane containing phenylhydrazine hydrochloride. It should be paraffined frequently.

*Glass plate for Visking membrane.* Photographic plates  $14 \times 3 \times 0.06$  inches are excellent for this purpose. The plates should be cut 1 inch wider than the test block.

*Glass container for phenylhydrazine solution.* A cuboidal glass container approximately  $4 \times 5 \times 3$  inches deep, with cover. A refrigerator dish is suitable.

*White opaque glass plate.* A white opaque glass plate  $4 \times 14 \times 0.25$  inches thick to be used as a background for matching color.

*Visking cellulose sausage casing.* A roll of Visking cellulose sausage casing 1.875 inches wide is used for the membrane (Visking Corporation, Chicago). This should be stored at room temperature.

##### *Reagents—*

*1 per cent phenylhydrazine hydrochloride.* 3 gm. of recrystallized phenyl-

<sup>2</sup> Formica was kindly supplied by the Formica Insulation Company, Cincinnati, Ohio.

hydrazine hydrochloride are dissolved in 300 cc. of distilled water (prepare fresh daily).

*10 per cent potassium ferricyanide.* 20 gm. of solid ferricyanide are dissolved in 200 cc. of distilled water.

*Approximately 3.5 N HCl.* 300 cc. of concentrated HCl are diluted to 1 liter.

*Standard Formaldehyde Solution*—A procedure for the standardization of concentrated (1 to 12 M) formaldehyde solutions has been previously described (5). Standard solutions (0.001 to 0.03 M) suitable for use in this diffusion procedure can be prepared by diluting standardized formalin solutions. Identical results are obtained by standardizing solutions prepared by hydrolysis and distillation of paraformaldehyde as described by Weinberger (6), or from commercial formaldehyde, the methyl alcohol of which is removed by refluxing, as described by Blair and Ledbury (7).

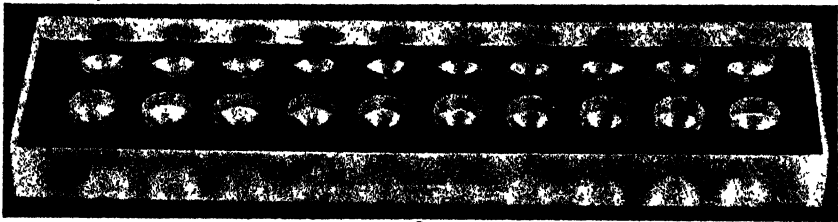


FIG. 1. Test block

If the pH of a solution to be tested lies between 3.0 and 7.0, the dilution of the standards can be made with distilled water. Addition of phthalate or acetate buffer in the range of pH 3.0 to 7.0 makes no difference in the results. Variations of salt concentrations to the extent of 1.0 M are without influence.

If the pH of a solution to be tested is above 7.0 or below 3.0, the pH of the standards must be adjusted to correspond with that of the unknown within 0.1 pH unit. Phosphate or borate buffers or sulfuric or hydrochloric acid added to make the solution 0.1 M may be employed to aid in maintaining a suitable pH.

The addition of 0.001 per cent sodium dodecyl sulfate to the standards facilitates pipetting the solutions so that they lie evenly in the depressions of the test block and with a meniscus approximating in shape that of many toxoids. Omission of dodecyl sulfate makes no difference in the results.

For testing toxoids dilute standards containing from 20 to 1000  $\gamma$  per cc., differing serially by 20  $\gamma$  per cc., may be employed.

Solutions buffered at pH 1.0 to 6.0 are stable for more than a week at 4°. Solutions buffered at pH 7.0 to 9.5 do not change for 24 hours at 4°.

*Procedure*—A small roll of Visking membrane (a sufficient amount for 1 day's use) is soaked in 1.0 per cent phenylhydrazine-hydrochloric acid solution in the glass container for at least a half hour before use. (The membrane may be left in the solution for the remainder of the day.) When the membrane is ready for use, it is pulled over the edge of the container onto a glass plate, so as to cover the entire length of the plate. The membrane is cut at the end of the plate and its surface is wiped with a clean cloth, thereby removing any adherent liquid, wrinkles, or bubbles of air which appear between the glass and the cellophane. The membrane is now ready for use.

1 cc. quantities of standard formaldehyde solutions and 1 cc. quantities of the unknown containing 20 to 1000  $\gamma$  per cc. are introduced into the holes of the test block. Standards are prepared so as to contain 20, 40, 60, 80, 100  $\gamma$ , etc., of formaldehyde per cc.

The plate membrane is placed over the holes in the test block. A small weight is put on the plate to hold it firmly against the block. The membrane is exposed for about 30 minutes for formaldehyde concentrations between 20 to 100  $\gamma$  per cc., 15 minutes between 100 and 200  $\gamma$  per cc., 5 minutes between 200 and 300  $\gamma$ , and 2 minutes for higher concentrations up to 1000  $\gamma$  per cc.

At the end of the exposure to the formaldehyde the plate membrane is removed from the test block and approximately 200 cc. of ferricyanide solution are poured on the exposed surface. The same quantity of 3.5 N HCl is poured on the membrane, and the red color develops almost immediately. In order to remove all excess ferricyanide the plate membrane is given a second washing with the same quantity of 3.5 N HCl. Excess reagent is wiped off and the glass plate with the membrane uppermost is placed against the white opaque glass background to match the unknown colors with the standard ones.

When many samples are to be tested of which the concentrations are not known even approximately, the following procedure saves time. All the samples are tested at the same time against standards which differ serially by 50 to 100  $\gamma$  per cc., depending on the probable spread of the concentrations of the unknowns. The membrane is exposed to the formaldehyde solutions in the block for 5 minutes. Samples of which the concentrations must be determined with greater accuracy are again tested against suitable standards which differ serially by 20  $\gamma$  per cc. and are exposed for the times suggested above.

*Preparation of Cellulose Nitrate Plate Films*—The following procedure for the preparation of plate films was employed in this laboratory for more than 9 months. The apparent intensity of color produced on these films

is at least twice as great as that produced on the cellophane membrane. 1 to 2  $\gamma$  of formaldehyde produces a readable color on the films. The procedure is therefore to be preferred for the estimation of concentrations of formaldehyde from 0 to 20  $\gamma$  per cc. An exposure time 50 to 75 per cent of that recommended for the cellophane plate is adequate and the color is developed as described for that procedure.

For the determination of free formaldehyde, in many toxoids the cellophane membrane is preferred because a minimum of skill is required in its preparation.

*Apparatus—*

*Turntable* (optional). A smooth board 14.5 inches long and 3.5 inches wide is mounted at its center on a vertical shaft in a bearing. The device is equipped with a brake. The surface of the board should be capable of rotation in a horizontal plane.

*Reagents—*

*10 per cent nitrocellulose.* A 10 per cent solution of nitrocellulose is prepared by dissolving 143 gm. of nitrocellulose (type 7720  $\frac{1}{2}$  second PXP containing 43 gm. of alcohol) in 457 cc. of aldehyde-free ethyl alcohol and 500 cc. of ether. This stock solution is stored in the cold room.

*0.6 per cent phenylhydrazine hydrochloride.* The phenylhydrazine hydrochloride is prepared by dissolving 3 gm. of recrystallized phenylhydrazine hydrochloride in 450 cc. of aldehyde-free ethyl alcohol and 50 cc. of ether. This solution is also kept in the cold room and should be prepared fresh each week.

*Nitrocellulose-phenylhydrazine mixture.* Equal quantities of 10 per cent nitrocellulose and 0.6 per cent phenylhydrazine hydrochloride are mixed in a graduate cylinder by inverting the cylinder several times. Approximately 30 cc. of this mixture are used in preparing each plate film. This mixture is to be prepared fresh each day.

Potassium ferrieyanide solution and 3.5 N hydrochloric acid are prepared as described for use with the cellophane membrane.

*Preparation of Plate Film—*Either of the following methods may be used satisfactorily.

1. A clean thin glass plate is placed in a horizontal position on a large level sheet of glass. With one stroke, the nitrocellulose-phenylhydrazine mixture is poured on the plate so as to cover it completely. The plate is immediately tipped upon its edge with the long axis still horizontal and allowed to drain 5 seconds. The plate is then laid flat on the level glass surface and permitted to dry for 3 minutes at 30° or 5 minutes at 25° in still air. When the plate film is made in this manner the unknowns and formaldehyde standards must be placed in the same row of holes in the test block.

2. A thin glass plate is placed with its center opposite the center of a

level turntable. A streak of beeswax at each end of the board will keep the glass in place. With one stroke the plate is covered with the nitrocellulose-phenylhydrazine mixture. The turntable is immediately spun so as to make two complete revolutions in approximately 1 second. Plate films made by spinning at double this speed are not satisfactory. After three or four revolutions the brake is applied. The plate is immediately placed on a level surface and dried for 3 minutes at 30° or 5 minutes at 25°. The colored spots produced by the same standards placed in both rows of holes in the test block will be identical for plate films prepared in this manner.

The prime objective in pouring the plate is to prepare a film which is uniform in thickness over the area which is to be exposed to the solutions. The technique employed should be tested by preparing films containing a dye, such as methyl red, and noting the uniformity of the film or by developing the color of the same standard placed in various holes in the test block.

The directions given above have worked satisfactorily at room temperatures from 25–30° and relative humidities of 60 to 75 per cent. Unusual atmospheric conditions might necessitate changes in the drying time. If the plate is too moist (too much solvent present), the color developed will be spotty. If the plate is dried too long, less than the usual amount of color will be developed. When the film is properly dried, the surface is still tacky and the odor of ether has for the most part disappeared.

*Effect of Added Substances on Diffusion of Formaldehyde*—It is shown (Table I) that redistilled acetone added to make the concentration 0.025 M, redistilled ethyl or methyl alcohol added to the extent of 0.5 M, or sodium chloride added to make the concentration 1.0 M did not influence the amount of color produced by a solution 0.01 M in respect to formaldehyde at pH 5.0 when compared with a 0.01 M formaldehyde solution at pH 5.0 without the additions. Similarly 0.01 per cent sodium dodecyl sulfate did not influence the results. Higher concentrations of the volatile substances decreased the color obtained. Concentrations of acetaldehyde greater than 0.001 M appreciably decreased the amount of color obtained. 20 cc. portions of solutions which were 0.1 M in respect to acetaldehyde and 0.01 M in respect to formaldehyde were aerated with a rapid current of air for 30 minutes and the solution made to its original volume. The original concentration of formaldehyde remained unchanged.

When the Schryver reagents are added to the solution to be tested, the resulting reaction is far from specific for formaldehyde (8). As used in this diffusion procedure, the reaction is more nearly specific. Compounds including lactic, pyruvic, malic, and tartaric acids, glycerol, and glycine, which are said to give rise to color when the reagents are added to the

solution (8), produce no coloration in the membrane used in the diffusion procedure.

*Effect of pH on Diffusion of Formaldehyde from Solution*—As shown in Fig. 2 the amount of color produced in the membranes in a given time by 0.01 M formaldehyde solution is constant if the pH is between 3.0 and 7.0. As the pH of the solutions is increased above 7.0 or decreased below 3.0, the amount of color produced in the membrane is increased. Similar results are given by 1.0 and 0.005 M solutions of formaldehyde.

To determine the influence of pH on the diffusion of formaldehyde, buffer solutions made according to Clark (9) were added to standard formaldehyde solutions so that the concentration of formaldehyde would be 1.0, 0.01,

TABLE I  
*Effect of Added Substances on Diffusion of 0.01 M Formaldehyde Solution*

Substance added	Concentration	Formaldehyde found
	M	$\gamma$ per cc.
0 . . . . .		300
Acetone . . . . .	0.025	300
“ . . . . .	0.5	280–290
Methyl alcohol . . . . .	0.5	300
“ . . . . .	1.0	280
Ethyl “ . . . . .	0.5	300
“ . . . . .	1.0	290
Acetaldehyde . . . . .	0.005	240
“ . . . . .	0.001	290–300
“ . . . . .	0.1 (Aerated 30 min.)	300
Sodium chloride . . . . .	1.0	300
“ . . . . .	0.5	300
	per cent	
Sodium dodecyl sulfate . . . . .	0.01	300

or 0.005 M in 0.1 M buffer solution. Comparable tests in 0.05 M buffers (above pH 1.0) gave similar results. 0.1 N hydrochloric acid, 0.1 N sulfuric acid (pH 1.0), phthalate (pH 2.4 to 5.6), acetate (pH 4.0 to 6.0), phosphate (pH 6.4 to 7.4 and 11.6), and borate (pH 8.6 to 9.6) buffers were used.

The color produced by the formaldehyde solutions at different pH values was compared with standard formaldehyde solutions buffered at pH 4.0, which differed serially by 20  $\gamma$  of formaldehyde per cc.

If the pH of a formaldehyde solution is changed from 1.0 or 9.0 to 4.0 or vice versa, the formaldehyde rapidly attains a rate of diffusion corresponding to the new pH.

The phenomenon is obviously a consequence of the dipolar characteristics of formaldehyde (10–15). The change in rate of depolymerization with the

change in pH found by Wadano, Trogus, and Hess (13) is similar to that which we have found in respect to the rate of diffusion.

It may be presumed that the change in diffusion with changes in pH is dependent on the extent of anionic or cationic dissociation of the formaldehyde. Whether or not that dissociation manifests itself by increased concentration of the ions in the surface layers or decreased polymerization in the surface layers, or both, has yet to be shown.

*Combination of Dilute Formaldehyde with Amino Acids*—The extent to which amino acids combine with dilute formaldehyde at different pH values was compared in the following way.

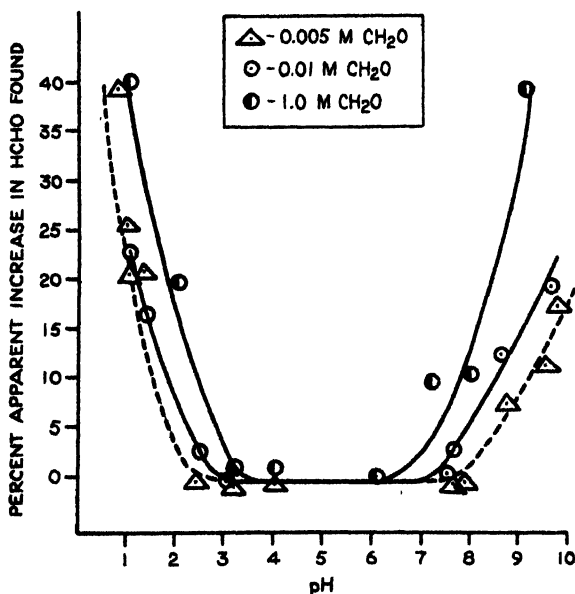


FIG. 2. Effect of pH on the diffusion of formaldehyde from solution

A series of formaldehyde standards which differed serially by 20  $\gamma$  per cc. from 20 to 300  $\gamma$  of formaldehyde was prepared in 0.1 M buffers at several pH values. Phthalate (pH 3.4 and 5.0), phosphate (pH 6.6, 7.4, and 11.6), and borate (pH 9.0) buffers were employed and prepared according to Clark (9). 0.1 N H<sub>2</sub>SO<sub>4</sub> was used for pH 1.0. The solutions contained 0.004 per cent of suitable indicators (thymol blue, brom-phenol blue, brom-cresol green, brom-thymol blue, phenol red, or tropaeolin OO).

Each amino acid-formaldehyde solution was prepared so that the concentration of amino acid was 0.1 M (or 0.01 M for the least soluble ones), formaldehyde 0.01 M (300  $\gamma$  per cc.), and buffer and indicator similar to that

in a series of formaldehyde standards. The pH of each was adjusted with NaOH or H<sub>2</sub>SO<sub>4</sub> to correspond, as judged colorimetrically, with that of the appropriate series of buffered formaldehyde standards.

The amino acid-formaldehyde solutions were compared against standards 1 hour and 24 hours after mixing. These reactions were carried out at room temperature (25°).

TABLE II

*Combination of 0.01 M Formaldehyde with Amino Acids at 25° during Period of 1 Hour after Mixing*

Each amino acid solution was 0.1 M with the exception of tryptophane and tyrosine. The total formaldehyde concentration of each solution was 0.01 M (300  $\gamma$  per cc.).

The results are expressed in micrograms per cc.

Amino acids	CH <sub>2</sub> O remaining in solution					
	pH 1.0	pH 5.4	pH 5.0	pH 7.4	pH 9.0	pH 11.0
Glycine . . . . .				300	300	20-40
dl-Alanine . . . . .				300	300	60-80
dl- $\beta$ -Phenylalanine . . . . .				300	300	280
dl-Valine . . . . .				300	300	150
l(-)-Proline . . . . .					300	
dl-Norleucine . . . . .				300	220*	
l(-)-Leucine . . . . .				300	200*	
l(-)-Tyrosine (0.01 M) . . . . .					300	280
l(+)-Glutamic acid . . . . .				300	220*	
l(+)-Aspartic " . . . . .				300	220*	
dl-Methionine . . . . .				300*	150	
l(+)-Lysine† . . . . .			300	300	70	
l(+)-Arginine† . . . . .				300	70	
dl-Serine . . . . .	280	280	280	240	50	
dl-Threonine . . . . .	270	250	240	90*	5-10	
dl-Tryptophane (0.01 M) . . . . .	280	240	140	210		
l(-)-Histidine† . . . . .	240	240	150	0		
l(-)-Cysteine† . . . . .	1-2	0	0			

\* Identical results were obtained 24 hours after mixing.

† Added as the monohydrochloride.

As is shown in Table II, a combination of formaldehyde and several amino acids which are not substituted in the  $\beta$  position is evident with the concentrations employed only at a pH above 9.0 or in some cases above pH 7.4. The reaction which occurs goes to completion promptly.

The extent of the reaction between formaldehyde and serine, threonine, and cysteine increased in the order given (Fig. 3; Table II). All three amino acids show some combination with formaldehyde below pH 7.0.



and in the case of cysteine essentially all formaldehyde was bound at pH 1.0.

The combination of tryptophane<sup>3</sup> with formaldehyde shows a maximum at pH 5.0 (Fig. 4; Table II). The reaction proceeds at a finite rate over several hours and finally results in the deposition of crystals. Histidine reacts rapidly with formaldehyde at pH 7.4. At pH 5.0 the reaction pro-

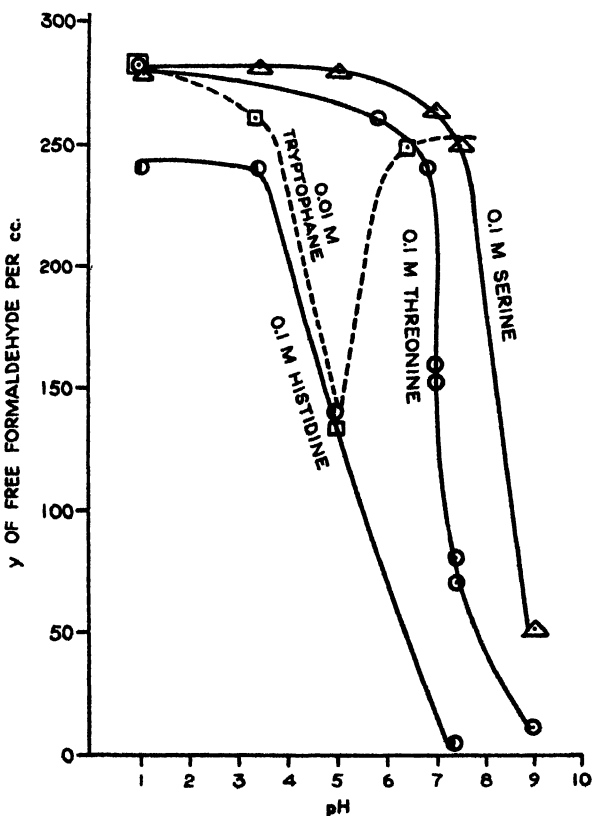


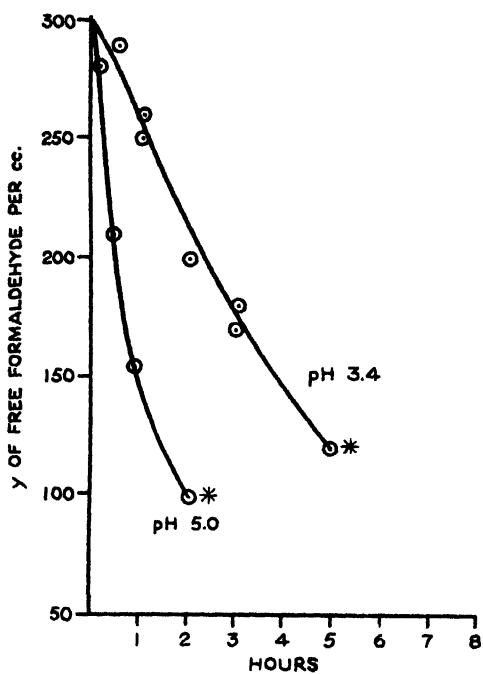
FIG. 3. Combination of amino acids with 0.01 M formaldehyde at different pH values 1 hour after mixing.

ceeds slowly for many days (Fig. 5) until with the concentrations employed the formaldehyde completely disappears from solution.

The results are consistent with and extend previous findings with serine (16), cysteine (17), tryptophane (18-20), and histidine (21).

In Fig. 6 is shown the disappearance of formaldehyde from a bacterial

<sup>3</sup> We are indebted to The Dow Chemical Company, Midland, Michigan, for a generous gift of synthetic *dl*-tryptophane.



\* Deposition of Crystals

FIG. 4. Combination of 0.01 M tryptophane with 0.01 M formaldehyde at different time intervals.

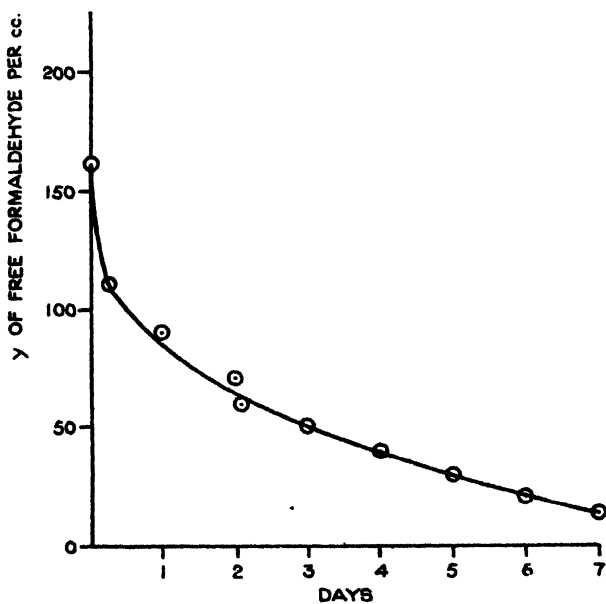


FIG. 5. Combination of 0.1 M histidine with 0.01 M formaldehyde at pH 5.0

filtrate as determined by this procedure. The filtrate was prepared from an enzymic digest of muscle which contained 0.4 per cent nitrogen. The conditions illustrated are typical but are not necessarily those found best suited for any particular detoxication. The loss of formaldehyde represents combination with constituents of the medium. Only an infinitesimal portion of formaldehyde can be presumed to combine with the toxin in these filtrates. In our hands the procedure has been of great value in controlling the concentrations of formaldehyde in bacterial filtrates from

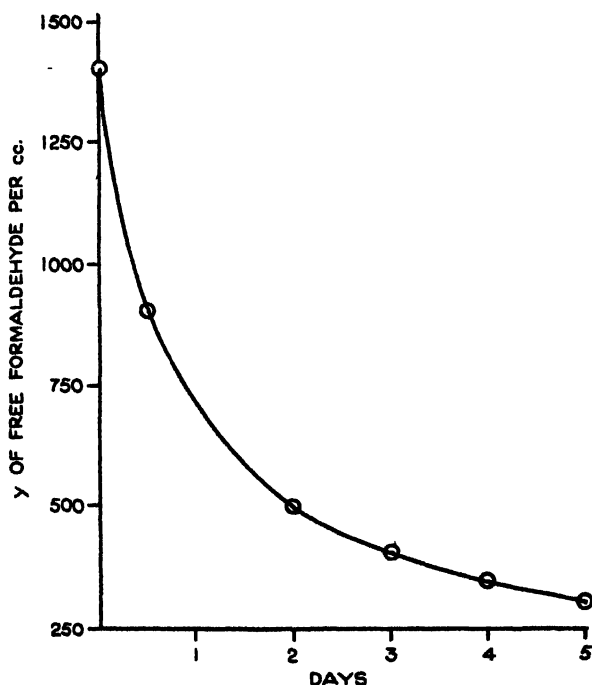


FIG. 6. Disappearance of formaldehyde from a bacterial filtrate

three different species in order to accomplish detoxication with minimum loss of antitoxin combining power.

#### SUMMARY

1. A procedure has been developed for the estimation of free formaldehyde in solution. The following principle is employed. Formaldehyde vapor is allowed to diffuse from solution into a membrane containing phenylhydrazine hydrochloride. A red color is developed in the membrane by adding ferricyanide and acid and is compared with colors developed at

the same time in the same manner from standard solutions of formaldehyde.

2. The effect of pH on the diffusion of formaldehyde from solution has been studied.

3. The reaction of dilute formaldehyde with amino acids at different pH values has been investigated. The results show that amino acids with SH or OH groups in the  $\beta$  position combine immediately to a greater extent with formaldehyde than those not so substituted.

4. The procedure is useful for the control of formaldehyde concentration during detoxication of bacterial filtrates.

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# PREPARATION OF PITUITARY THYROTROPIC HORMONE\*

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The existence of a thyroid-stimulating hormone in anterior pituitary gland tissue has been known for 30 years (1, 2). During this time, considerable work has been done on the physiological and the pharmacological effects of the thyrotropic hormone administered in the form of pituitary extracts which had been subjected to a varying degree of manipulation. Relatively little progress on the purification and chemical characterization of the thyrotropic hormone was made until 1931 (3). Since that time several methods of purification of the pituitary thyrotropic hormone have been described. Although some of these methods (4-9) effect significant concentration of the thyrotropic activity of pituitary tissue, the procedures either are not described in sufficient detail to allow ready repetition or result in poor yield of hormone. The most active preparation so far reported appears to be that of Fraenkel-Conrat, Fraenkel-Conrat, Simpson, and Evans (9), whose method yields material assaying 480 chick weight units per mg. of nitrogen. This represents a 100-fold increase in concentration of the hormone. Since their preparation contained 13 per cent N, it would assay about 65 chick weight units per mg. on a dry weight basis.

In no case has more than a crude attempt been made to characterize the chemical and physicochemical properties of the hormone. Available evidence indicates that the hormone is protein in nature.

It is the object of the present communication to present a description of a rather simple procedure which consistently yields significant amounts of a thyroid-stimulating product with a degree of physiological activity higher than any preparation previously described in the literature. A brief qualitative description of the thyrotropic preparation is given. At

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Some of the data in this paper are taken from a dissertation presented by Leon S. Ciereszko to the faculty of the Graduate School of Yale University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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a later time, the physicochemical properties of the product will be described.

In a previous publication from this laboratory, Bonsnes and White (10) described a procedure for the fractionation of saline extracts of fresh beef pituitary glands. By the use of isoelectric precipitation, followed by gradually increasing concentrations of acetone, it was possible to prepare a fraction with marked thyrotropic hormone activity. Essentially, this fraction represented material precipitated from a saline extract of pituitary tissue by 75 per cent acetone at pH 4.0 after the previous removal of precipitates at pH 5.4, 4.8, 4.0, and 4.0 with the addition of acetone to a concentration of 50 per cent.

The preparation of the thyrotropic fraction of Bonsnes and White has been simplified by omitting the isoelectric precipitations at pH 5.4 and 4.8. Thyrotropic hormone activity has been further concentrated by extraction of the 75 per cent acetone-insoluble precipitate with water, and treatment of this aqueous extract with lead acetate and with trichloroacetic acid. The product obtained from whole frozen beef pituitary glands by this procedure gives a positive histological response in the thyroids of white Leghorn chicks when given at a total dose of 1  $\gamma$ . Growth, gonadotropic, and prolactin activities are absent from the preparations obtained from beef glands. Preliminary studies in the Tiselius electrophoresis apparatus and in the analytical ultracentrifuge indicate the homogeneity of the preparation.

#### EXPERIMENTAL

*Method of Assay*—The biological activities reported in this study are based on the histological changes in the thyroid of the 8 day-old male white Leghorn chick. Injections were started when the chicks were 3 days old. Single, subcutaneous doses were given daily for 5 days, a volume of 0.5 ml. being used for each injection. 24 hours after the last injection, the chicks were killed with illuminating gas and the thyroids carefully dissected out and weighed. The adrenals and gonads were also weighed at this time. The thyroids were then prepared for histological examination in the usual manner. The minimum effective dose, *i.e.* the minimum amount of the substance required to produce a definitely positive histological response, was determined for each preparation. The histological picture observed microscopically is similar to that in the photographs published recently by Jorgensen and Wade (11). Each dose level has been assayed on a minimum of eight chicks, and each assay was always accompanied by a group of eight uninjected control birds. The histology of the latter group served as a control for possible variations in chicks and in environmental conditions. White Leghorn chicks have been used

exclusively, and the birds were always purchased from the same hatchery.<sup>1</sup> Environmental conditions were controlled by the use of constant temperature brooders. The non-stimulated histological appearance of the thyroids of the control chicks has been quite uniform and has made possible consistent assay results. Questionable stimulation has always been put in the negative group, and only definite, actual changes in the cells, with some resorption of the colloid and few resting interstitial cells, have been graded as a +1 or a minimum response.

A few comments are necessary regarding the chick assay, which is based on the work of Smelser (12). During the course of several years of investigation in this laboratory on the thyrotropic hormone, several thousand chick thyroids have been weighed and subjected to histological examination. At intervals 1, 2, 3, and 4 day-old chicks have been used for comparative assays. The 3 day-old chick was finally selected for two reasons: (1) a high mortality in the younger injected birds was generally encountered, and (2) a resting thyroid in the control non-injected chick was uniformly and consistently obtained when the chicks were permitted to adjust themselves to their new environment for a few days after arrival from the hatchery.

It may be added that little success attended efforts to use thyroid weight increase as a measure of the activity of thyrotropic hormone. Although careful dissection of the thyroids has been regularly conducted, it has not been possible to obtain a significant correlation between gland weight and histological response. It has been a very common experience to obtain definite histological alterations in some instances in which there were no changes in the weight of the thyroids. In other birds, thyroid weight increases were found without histological changes. Therefore, histological response alone was considered an accurate indication of thyrotropic hormone activity.

For purposes of convenience in the presentation of the data, the minimum amount of a preparation required to produce a definite histological response, under the conditions of the assay described above, is termed 1 unit.

*Method of Preparation*—In so far as possible the procedure is carried out at 3–5° with cold solvents and solutions. All pH measurements were made with the Beckman glass electrode.

1 kilo of frozen whole beef pituitary glands is ground finely in a motor-driven meat grinder. 5 liters of 2 per cent sodium chloride solution are added to the mash. The mixture is thoroughly agitated by means of mechanical stirring and the pH adjusted to 7.4 to 7.8 by the addition of about 50 ml. of 2 N NaOH. The extraction is conducted with continuous stirring for 3 to 4 hours. The tissue residues are removed by centrifuging

<sup>1</sup> Kerr Chickeries, Frenchtown, New Jersey.



and the turbid supernatant acidified to pH 4.0 to 4.1 with 2 N HCl. The precipitate is then removed immediately by the use of the continuous Sharples supercentrifuge. To the supernatant (pH 4.0 to 4.1) an equal volume of acetone is added and the mixture allowed to stand overnight. The supernatant solution is siphoned off, clarified by filtration, and to it an equal volume of acetone is added. The precipitate which forms is allowed to settle out overnight. The supernatant acetone solution is siphoned off, and the precipitate is centrifuged and washed with a mixture of 3 parts of acetone and 1 part of water by dispersing the precipitate thoroughly in the aqueous acetone and centrifuging. The washing is repeated three times. The washed precipitate is then dried by triturating with acetone and centrifuging. The acetone washing is repeated three times. Finally, the precipitate is stirred up with ether, centrifuged, and put in a desiccator under a vacuum. After about 15 minutes, the precipitate is thoroughly broken up with a small spatula and replaced in the desiccator. The vacuum is turned on and released several times, the precipitate being broken up finely each time until the product is a fine dry powder. The yield is 5 to 6 gm. (Fraction CA).

An aqueous extract is then made by stirring the dry precipitate successively with one 100 ml. portion and three 50 ml. portions of distilled water in a centrifuge bottle for 5 to 10 minute periods. Each extraction mixture is centrifuged and the supernatant solutions are filtered and combined. The insoluble residue from the last extraction is discarded. The clear buff-colored extract obtained in this manner has a pH of approximately 7. A precipitate appears upon the addition of 1 N NaOH to pH 9. This precipitate is centrifuged off and the pH of the supernatant solution adjusted to 7.0. A solution of 5 per cent lead acetate ( $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ ) is added until precipitation is complete. The amount of lead acetate solution required is determined on small measured samples of the water extract. After the addition of lead acetate, the solution is allowed to stand in the refrigerator until the precipitate settles out, leaving a clear supernatant. This takes less than an hour. After removal of the lead precipitate at the centrifuge, the pH of the supernatant varies from 5.0 to 5.4.

To the lead acetate supernatant, 20 per cent (1.25 M) trichloroacetic acid is added slowly and with stirring until a concentration of 8 per cent (0.5 M) trichloroacetic acid is obtained. The white precipitate which appears is allowed to flocculate by standing in the refrigerator for 1 hour and is then removed by centrifugation.

The clear and colorless supernatant, obtained after removal of the trichloroacetic acid-insoluble precipitate, has a pH of about 1.2. This solution is dialyzed in viscose tubing against running cold water until all

trichloroacetic acid has been removed. The dialyzed solution is then concentrated by pervaporation to a volume of 75 to 100 ml. and lyophilized. About 400 to 500 mg. of a white solid designated as Fraction SPbT are thus obtained from 1 kilo of frozen whole beef pituitaries.

The material obtained by the procedure described above produces histological changes in the thyroids of 3 day-old chicks injected over a period of 5 days with a total dose of 1  $\gamma$ . It gives the usual protein color tests and is very soluble in water. The Molisch reaction is positive. Preliminary electrophoretic examination of the product, as well as ultracentrifuge studies, suggests that it contains but one protein component. The nitrogen content is 12.6 per cent; sulfur is 1.2 per cent. Tests for phosphorus are negative. Sulfosalicylic acid fails to precipitate the thyrotropically active protein; it is precipitated by phosphotungstic and picric acids, uranium acetate, and mercuric chloride. The lead used in the purification procedure is removed during the dialysis. Tests on the final product with hydrogen sulfide and dithizone indicate the absence of lead.

The thyrotropic hormone prepared by the method described above has been examined for other types of anterior pituitary activity. Growth-promoting action was tested in four hypophysectomized rats by injecting the substance into each animal in doses of 5 mg. daily for a 7 day period. There was no increase in body weight in any of the rats during the injection period.

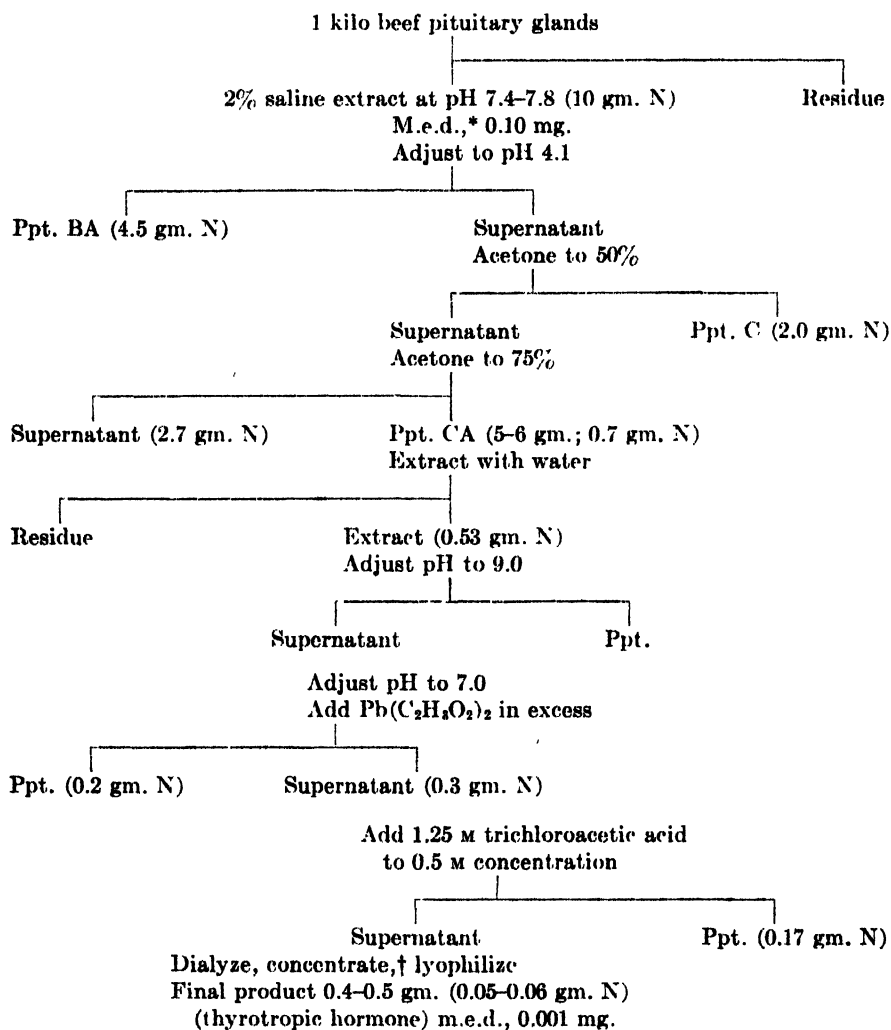
Prolactin activity has been determined by the systemic method of Lyons (13), employing 6 week-old white Carneau pigeons. A total dose of 5 mg. produced no evidence of prolactin activity.

Gonadotropic activity has been evaluated throughout this study by determining the effect of various preparations on the weights of the testes of the same chicks employed in the thyrotropic assays. Although no effort has been made to determine variations in gonad weight as a measure of gonadotropic activity, certain conclusions are permissible from the many hundreds of chick gonads which have been weighed. The most striking and consistent observation has been the lack of evidence of definite gonadotropic potency in the beef thyrotropic preparation described above. Dr. W. U. Gardner of the Department of Anatomy has kindly examined the effect of the thyrotropic preparation on the gonads of the male hypophysectomized mouse, and reports no evidence of gonadotropic activity.

#### DISCUSSION

It was found that the fractionation procedure of Bonsnes and White (10) may be shortened without affecting the yield of the thyrotropic fraction. This was done by omitting the isoelectric precipitations at pH 5.4 and 4.8. The thyrotropic fraction (CA) represents less than 10 per cent

of the nitrogen originally present in the saline extract of the pituitary glands. The distribution of nitrogen in the course of the fractionation procedure is indicated in the accompanying flow chart.



\* Minimum effective dose in chick assay.

† Solution concentrated in dialysis bag by pervaporation.

A constant fraction of the nitrogen in Precipitate CA is extracted by water. In four different experiments water extraction of various CA fractions yielded 92.4, 91.6, 92.5, and 89.0 mg. of nitrogen per gm. of

Fraction CA extracted. The water-extracted residue was inactive when assayed for thyrotropic activity.

Since thyrotropic activity began to appear in the precipitate at 30 per cent acetone from the water extracts of Fraction CA, the question arose as to whether significant loss of the hormone had occurred at the step just prior to removal of Fraction CA; that is, in the fraction of the saline extract insoluble at pH 4.1 in 50 per cent acetone (Fraction C). Accordingly, the acetone-dried Fraction C was extracted with water, the extracted material precipitated by the addition of 3 volumes of acetone to the water extract, and the precipitate assayed. The material derived in this way from Precipitate C showed activity only at high dose levels in comparison with that from Fraction CA. In addition, it could be obtained only in small amounts, indicating that little thyrotropic activity was lost by the removal of Precipitate C in the fractionation of saline extracts.

Water extracts of Fraction CA, to which 5 per cent lead acetate solution has been added, still contain protein nitrogen after removal of the lead precipitate. Furthermore, it has been demonstrated that these supernatants from the lead precipitation retain the thyrotropic activity of Fraction CA, while the protein which can be recovered from the lead precipitate by extraction with dilute disodium hydrogen phosphate solution is thyrotropically inert.

Addition of trichloroacetic acid to the lead acetate supernatants precipitates about 55 per cent of the nitrogen present. The proportion of the nitrogen precipitated seems to be independent of the nitrogen concentration. For example, of 0.45, 0.90, and 1.23 mg. of N per ml. in lead acetate supernatants, 45, 45, and 46 per cent remained in solution after the addition of trichloroacetic acid to 0.5 M concentration. About half of the nitrogen in the trichloroacetic acid supernatant is removed by dialysis.

The yields of thyrotropic hormone obtained by the procedure here presented indicate that a good recovery of the hormone originally present in the saline extracts of pituitary tissue is obtained in the trichloroacetic acid-soluble fraction. The saline extract obtained from 1 kilo of frozen whole beef pituitaries contains about 62.5 gm. of protein material ( $N \times 6.25$ ) and represents about 625,000 units of thyrotropic activity. The total yield of the thyrotropic protein fractions is about 500 mg. with a minimum effective dose of 1  $\gamma$ . The yield of thyrotropic activity is thus about 500,000 units.

Thyrotropic preparations of equally high potency have been obtained from dissected sheep anterior lobes with the above procedure; however, these preparations always contain gonadotropic material. Beef glands contain relatively little gonadotropic hormone and yield a thyrotropic hormone preparation which is free from gonadotropic activity.

## SUMMARY

Marked purification of the pituitary thyrotropic hormone has been achieved by a relatively simple procedure which yields good recoveries of thyroid-stimulating material from frozen whole beef pituitaries. 1  $\gamma$  of the thyrotropic preparations obtained by this procedure produces definite histological changes in the thyroids of 3 day-old male white Leghorn chicks. The preparations do not have demonstrable prolactin, gonadotropic, or growth hormone activity.

I wish to express my appreciation to Dr. Abraham White, who suggested this problem, for his encouragement and sound guidance, and to Miss Hilda Ritter, who carried out the large number of chick assays which guided this work.

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# A METHOD FOR THE DETERMINATION OF COPPER IN BLOOD SERUM\*

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The demonstration that copper is transported in the serum (1) makes available a means of studying copper metabolism and gives rise to the necessity of having a simple and accurate method which can be applied to the measurement of a large number of samples. The purpose of this paper is to present such a method.

Sodium diethyldithiocarbamate has been found to be a satisfactory and sensitive reagent for the determination of copper. Callan and Henderson (2) discovered that when this substance was added to a solution of copper a golden brown color was produced. McFarlane (3) found that the colored copper salt could be rapidly and quantitatively extracted from aqueous solution by amyl alcohol and that the color was intensified in the organic solvent. The depth of color was reported to be directly proportional to the amount of copper present, provided the range of copper concentrations was not too great. The color complex is stable for at least 2 hours and the pH of the solution has little effect on the color intensity between pH 5.7 and 9.2. Iron gives a brown color with the reagent and is the only substance in biological ash which is known to interfere significantly. However, when sodium pyrophosphate is added, iron pyrophosphate is formed and this compound does not react with the carbamate, whereas the reactivity of the copper is unaffected (3, 4).

Locke, Main, and Rosbash (5) prepared a protein-free filtrate of serum by precipitating the proteins in the cold with trichloroacetic acid and determined the copper content of the filtrate with the carbamate reagent. Recoveries of added copper were not reported. Tompsett (6) demonstrated that the whole of the serum copper is present in such a filtrate and is present in such a form that it can be determined directly with carbamate. Using such a procedure, he obtained excellent recoveries and the results compared closely with those obtained by ashing. The observations of Yoshikawa, Hahn, and Bale are not in agreement (7). Using radioactive copper, they found that nearly all of the copper in plasma is bound in some manner to

\* The work described in this paper was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Utah.

protein and that only about two-thirds is split off with trichloroacetic acid in the cold.

McFarlane (3) as well as Sachs, Levine, Anderson, and Schmit (4) has determined serum copper with the carbamate reagent by dry ashing first. However, ashing methods are fraught with many difficulties such as spattering, contamination from reagents, and volatilization of the copper if the temperature is too high. They are inconvenient, time-consuming, and require a muffle furnace or a digestion rack. Such methods do not lend themselves well to the determination of a large number of specimens. Therefore, it was felt that if a reliable method could be devised which avoided ashing and was relatively simple it would be well worth while.

#### EXPERIMENTAL

Standard copper solutions with amounts of copper varying from 1 to 10  $\gamma$  per 10 ml. were treated according to the method of Locke, Main, and Rosbash (5) with 0.2 ml. of pyridine and 1 ml. of 1 per cent carbamate and extracted with 10 ml. of amyl alcohol. The alcoholic extracts were then read in the Evelyn photoelectric colorimeter with the 6 ml. aperture and Filter 440. The results are presented in Fig. 1. The extracts were cloudy and first had to be filtered, but even with this additional step results were not reproducible and a reliable standard curve could not be obtained. This can be explained by the fact that in order to read in the macro Evelyn colorimeter it was necessary to extract with at least 10 ml. of alcohol and as a result the final concentration of copper was not great. McFarlane (3) used larger concentrations of copper and smaller volumes of amyl alcohol, and measured the color intensity of a Duboscq colorimeter with micro cups. Locke, Main, and Rosbash (5) used only 2 ml. of alcohol and made crude colorimetric comparisons with a standard held in the sunlight. These differences probably account for the variability of the results of the amyl alcohol extractions. In any event, it was decided that this procedure was not suitable for use in the Evelyn colorimeter under these conditions.

In Fig. 2 the results of reading the color directly in the aqueous solution are shown. 1 ml. of a saturated solution of sodium pyrophosphate, 2 ml. of redistilled ammonium hydroxide, and 1 ml. of a 0.1 per cent solution of sodium diethyldithiocarbamate were added to 10 ml. of varying dilutions of a standard copper solution. The volume was then made up to 15 ml. with redistilled water and the solutions read in the Evelyn colorimeter with the 10 ml. aperture and Filter 440. As can be seen, the results were considerably more consistent than with the amyl alcohol extraction procedure. It was therefore decided to read the color directly in the aqueous solution and to avoid the additional as well as unnecessary and unreliable step of extracting with amyl alcohol.

To ascertain what portion of the total serum copper is present in a trichloroacetic acid filtrate 5 ml. samples of serum, to which known amounts of copper were added, were pipetted into 15 ml. Pyrex centrifuge tubes and 3 ml. of redistilled water added. The tubes were then placed in a water bath at boiling temperature until the solutions became opaque, were cooled, and following the addition of 2 ml. of 20 per cent trichloroacetic acid were placed in a water bath at 90-95° for 3 minutes with stirring. The tubes

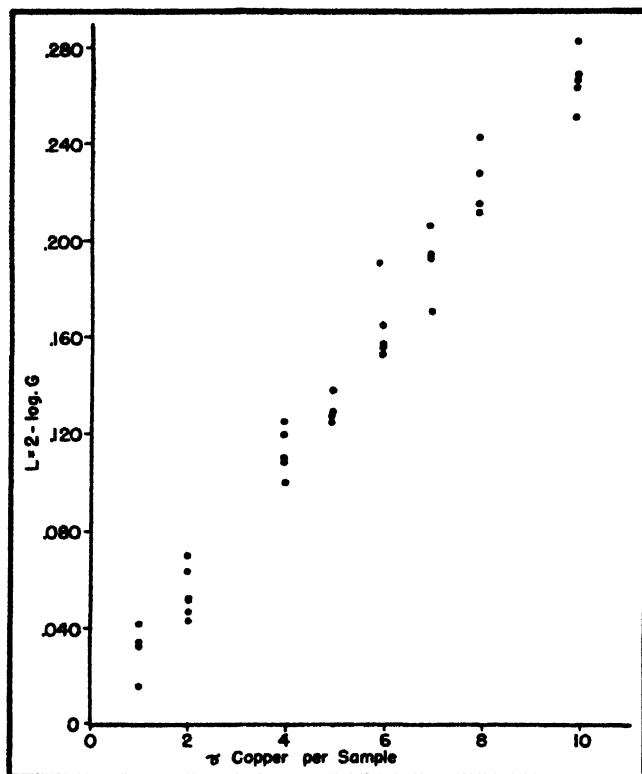


FIG. 1. Showing the variability of amyl alcohol extractions of standard copper solutions.  $L$  refers to photometric density.  $G$  is the galvanometer reading.

were then spun at 2500 R.P.M. for 15 minutes. The supernatant solutions were decanted and the copper content determined in the aqueous solution with carbamate as described above. An average of 74 per cent of the added copper was recovered, as can be seen in Table I. Following resuspension of the original precipitate and a second extraction, it was possible to recover a total of 89 per cent. Three extractions yielded an average recovery of 97 per cent.



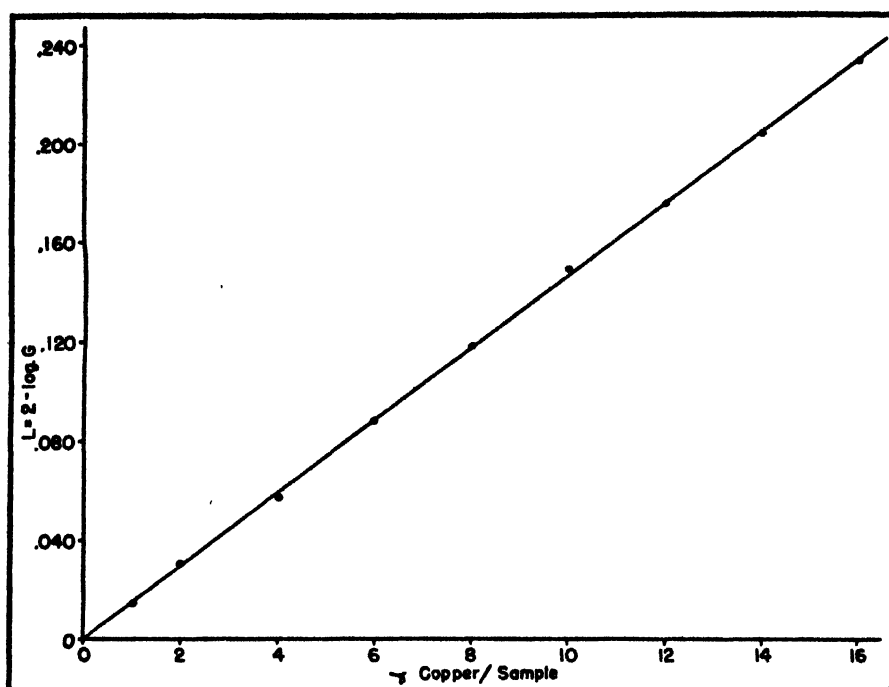


FIG. 2. The standard curve obtained by adding sodium diethyldithiocarbamate directly to the aqueous copper solution.  $L$  refers to photometric density.  $G$  is the galvanometer reading.

TABLE I

*Showing Average Recovery of Copper following One, Two, and Three Extractions of Trichloroacetic Acid Precipitate*

Experiment No.	No. of trichloroacetic acid extractions	No. of determinations	Average recovery
			<i>per cent</i>
I	1	4	74
II	2	10	89
III	3	24	97

### *Proposed Method*

#### *Reagents—*

1. Copper-free water. Redistil water in an all-glass distilling apparatus.
2. Trichloroacetic acid, 20 per cent in redistilled water. The trichloroacetic acid is first distilled in an all-glass apparatus to free it of copper.
3. Sodium pyrophosphate. Make up a saturated solution in redistilled water.

4. Ammonium hydroxide, approximately 28 per cent. Distil reagent grade ammonium hydroxide through an all-glass apparatus into redistilled water until the solution is saturated.

5. Sodium diethyldithiocarbamate (Eastman). Make up a 0.1 per cent solution with redistilled water.

6. Standard solution of copper. Dissolve 0.3928 gm. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in redistilled water. Dilute to 1 liter. (It is important to use clear crystals of copper sulfate that show no sign of efflorescence.) Make up working solutions from this.

All glassware used must be carefully cleaned and then rinsed three times with redistilled copper-free water.

Pipette 3 to 5 ml. samples, preferably 5 ml., of serum or plasma into ungraduated 15 ml. Pyrex centrifuge tubes. It is convenient to set up that number of tubes which can be centrifuged simultaneously. Add 1 ml. of glass-redistilled water to each, mix with glass rods, and place the tubes in boiling water. When the solutions become opaque, remove the tubes and cool. Add 2 ml. of 20 per cent trichloroacetic acid, stir thoroughly, and heat in the water bath at 90–95° for 5 minutes. Stir frequently while heating. Cool as before, remove the rods, and centrifuge the tubes at 3000 r.p.m. for 10 minutes. Decant the supernatant solutions into 15 ml. graduated centrifuge tubes.

To the original tubes add 1 ml. of trichloroacetic acid and 1 ml. of water, break up the precipitates well with the glass rods, and while stirring heat again in the water bath at 90–95° for 5 minutes. Remove, cool, and centrifuge as before. Decant the supernatant fluid into the tubes containing the first filtrate.

Again add 1 ml. of trichloroacetic acid and 1 ml. of water to the precipitates, treat, centrifuge, and decant as before.

To the graduated tubes containing the three filtrates add 1 ml. of a saturated solution of sodium pyrophosphate, 2 ml. of ammonium hydroxide, and 1 ml. of a 0.1 per cent solution of sodium diethyldithiocarbamate. Make the volume up to 15 ml. with redistilled water. Mix the solutions well by pouring back and forth between the centrifuge and colorimeter tubes. Read as soon as possible in the Evelyn photoelectric colorimeter, using Filter 440 and a reagent blank to set the galvanometer at 100.

Calculate the copper content of the solutions, using the formula

$$L \times \frac{100}{\text{ml. serum used}} \times \frac{1}{K} = \text{copper, micrograms \%}$$

$L$  refers to the photometric density and is calculated from the galvanometer reading  $G$  by the formula,  $L = 2 - \log G$ .  $K$  is the calibration constant.

The value of  $1/K$  as established on our instrument is 67.6.

Twelve representative recoveries with the method outlined above are presented in Table II. The method not only gives excellent recovery of added material but offers results which are consistently reproducible within  $\pm 10$  per cent.

TABLE II

*Results of Recovery of Copper following Addition of Known Amounts to Serum*

Experiment No.	Serum	Added	Total	Found	Recovery
	$\gamma$ per cent	$\gamma$ per cent	$\gamma$ per cent	$\gamma$ per cent	$\gamma$ per cent
1	88	43	131	125	95
2	88	43	131	127	97
3	88	43	131	129	98
4	88	43	131	122	93
5	88	85	173	163	94
6	88	85	173	165	95
7	88	85	173	165	95
8	88	85	173	169	98
9	102	142	244	246	101
10	102	142	244	246	101
11	103	47	150	149	99
12	103	47	150	149	99
Average.....					97

TABLE III

*Range of Serum Copper Values as Obtained by Various Authors*

Author	Method	Serum copper
		$\gamma$ per cent
Locke <i>et al.</i> .....	Trichloroacetic acid	72- 95
Tompsett.....	" "	183-245
Guillemet and Schell.....	Dry ashing	56- 75
Warburg and Krebs.....	Catalytic	82
Sachs <i>et al.</i> .....	Dry ashing	82-132
Present method.....	Trichloroacetic acid	92-150

There is a small error incurred by the use of the Evelyn colorimeter with colored solutions of low intensity. Attempts were made to eliminate this error by concentrating the filtrates, by using smaller volumes of reagents, and by combining reagents. None of these methods proved practical. However, the excellent recoveries and reproducibility of results obtained with the method outlined justify this error.

In Table III the range of serum copper for normal adults as obtained by

various authors is summarized. Locke, Main, and Rosbash (5) do not mention filtering the cloudy amyl alcohol and used a crude comparator. They made twenty-eight determinations on "normal" adults but included only seventeen of the determinations in the average. The determinations were made following one trichloroacetic acid precipitation. The values reported by Tompsett (6) on eight individuals are extremely high. The cause for this is not apparent. His values for total blood copper are also out of line with those obtained by others (4). Guillemet and Schell (8) obtained low values for serum copper. Tompsett (6) states that the number of precipitations used in their method probably accounts for the low values obtained. Warburg and Krebs (9), using a method based on the

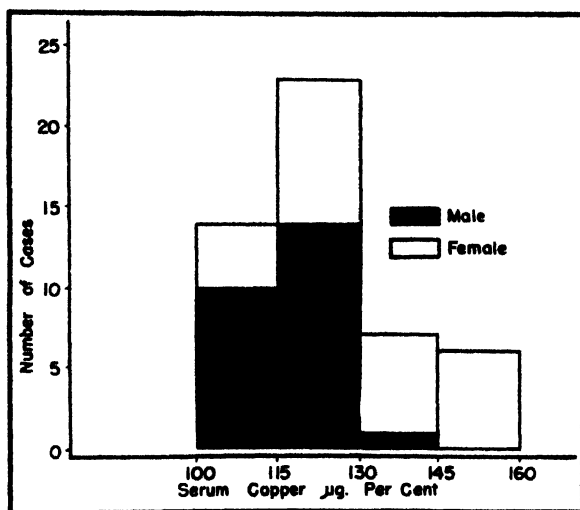


FIG. 3. The copper content of the serum of twenty-five normal males and twenty-five normal females.

fact that copper catalyzes the oxidation of cysteine to cystine, reported 0.082 mg. per 100 ml. as their average for ten normal adults. Sachs, Levine, Anderson, and Schmit (4) determined the copper content of the serum of ten adult males, using a dry ashing procedure followed by carbamate, and reported an average of 0.105 mg. of copper per 100 ml. of serum.

We have determined the copper content of the serum of twenty-five healthy adult males and twenty-five healthy adult females. The results are presented in Fig. 3. The average value for males was 116  $\gamma$  per cent. The lowest value obtained was 92  $\gamma$  per cent, and the highest 134  $\gamma$  per cent. These values correspond well with those obtained by Sachs *et al.* (82 to 132  $\gamma$  per cent) (4). For the females the average was somewhat higher, 131  $\gamma$  per cent. The values ranged from 103 to 159  $\gamma$  per cent.

## SUMMARY

1. Evidence has been presented that approximately 75 per cent of the copper contained in serum is present in the filtrates prepared by precipitation of the serum with trichloroacetic acid. Three warm extractions of the trichloroacetic acid precipitate have been shown to remove approximately 97 per cent of the copper.

2. A method for the determination of copper in the serum or plasma has been presented which is based on a triple warm extraction of a trichloroacetic acid precipitate followed by the colorimetric determination of copper with sodium diethyldithiocarbamate. The colored solutions were read in the Evelyn photoelectric colorimeter.

3. Values of serum copper for twenty-five normal males and twenty-five normal females are presented.

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## GROWTH AND LIPOTROPISM

### I. THE DIETARY REQUIREMENTS OF METHIONINE, CYSTINE, AND CHOLINE

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Since the reviews by McHenry and Patterson (1) and Best and Lucas (2) on the relationships of lipotropic substances to fat metabolism, several publications have appeared which indicate progress in clarifying the somewhat conflicting views regarding the influence of amino acids and proteins on the fat content of the liver. Beveridge and coworkers (3) have shown that certain essential amino acids have a marked effect on the lipotropic efficacy of methionine. They also found no significant difference in the lipotropic effect exerted by methionine when fed as the amino acid or in casein, provided the essential amino acids were approximately equalized in the two diets. The data of Beveridge and coworkers give strong support to the explanation proposed by Treadwell, Groothuis, and Eckstein (4) for the greater lipotropic activity of free methionine in comparison with protein-contained methionine. It was suggested that larger amounts of methionine were used for the synthesis of tissue protein in the casein-fed groups, thus producing a decrease in the amount available for lipotropic activity with a resultant higher level of liver fat. The basal diet (4) contained 5 per cent casein, which is insufficient to support normal growth in young rats irrespective of the level of supplementary methionine. Horning and Eckstein (5) found that with adult male rats (250 gm.) a diet containing 5 per cent casein was, as far as lipotropism is concerned, as effective when supplemented with methionine and cystine as when supplemented with sufficient amounts of cystine and casein to equalize the content of the sulfur-containing amino acids in the two diets. The level of liver fat was low in all the groups receiving supplements, in marked contrast to results with young animals (4) in which the liver fat was low only in those receiving amino acid supplements. In these animals, as the authors (5) point out, the protein and methionine requirements for growth were low, so that a considerable part of the methionine in the supplements was available for lipotropic action. Treadwell, Tidwell, and Gast (6) have extended observations on young rats (130 gm.) to include the effect of diets so designed that the protein and methionine levels could be varied independently of each

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other. The data indicated a preferential utilization of methionine for growth when there were adequate amounts of the other essential amino acids in the diet. However, additional methionine over that used for growth produced a definite decrease in liver fat. Thus, the recent data from three laboratories support the explanation given by Treadwell *et al.* (4) for the difference observed in the lipotropic activity of free and protein-contained methionine.

Handler (7) has concluded that the fatty livers due to deficiency of choline develop only when all other dietary factors are present in sufficient concentration to permit at least slow growth. The data upon which this conclusion was based were obtained with diets which contained sufficient protein so that when growth was decreased owing to a deficiency of some essential factor sufficient methionine became available from the dietary protein to exert a definite lipotropic effect. Thus, in the experiments (7) on the effects of simultaneous mineral and choline deficiencies on liver fat, when the animals' mineral supply was exhausted there were a decline in weight and a lowering of the liver fat toward normal. We suggest that the liver fat decreased because the methionine (455 mg. per cent) in the dietary casein (15 per cent) was not entirely used for growth while the animals were declining in weight and therefore a part was available for lipotropic action.

The findings discussed above suggested a possible division of the total methionine requirement into two components; namely, the methionine required for growth and that required for lipotropism. It seemed likely that the magnitude of these two components would be influenced by the amounts of other substances present in the diets, such as amino acids, fats, vitamins, minerals, choline, and related substances. Also the stage of the life cycle in which the requirement is determined is important as is indicated by the work of Horning and Eckstein (5). We have obtained data concerning certain of these points which will be published later. In the study presented below we have investigated the growth and lipotropic requirements for methionine, cystine, and choline in rats receiving diets which contained adequate protein, minerals, and vitamins for optimum growth during the experimental period. The basal choline-free diet supplied 500 mg. of methionine and 100 mg. of cystine per 100 gm. of diet, which were the amounts found by Womack and Rose (8) to be necessary for optimum growth.

#### EXPERIMENTAL

White male rats weighing approximately 170 gm. (range 160 to 180) of the Sprague-Dawley strain were used. The care and environment of the animals were the same as described previously (9). The basal diet

(No. 26, Tables I to III) consisted of 15.4 per cent casein, 3.2 per cent arachin, 5 per cent salt mixture (10), 2 per cent Cellu flour, 34.4 per cent glucose,<sup>1</sup> and 40 per cent lard. The casein was a commercial vitamin-free product. The arachin was prepared from peanut flour<sup>2</sup> by the method of Johns and Jones (11). The proteins were heated at 98° until the moisture content was less than 1 per cent before incorporation into the diets. The mixture of casein and arachin in the basal diet was calculated to provide 500 mg. of methionine and 100 mg. of cystine. The methionine value was confirmed by analysis according to the directions of Albanese *et al.* (12). The various dietary constituents supplied a total of not more than 1 mg. of choline per 100 gm. of diet. When supplementary methionine, cystine, or choline was incorporated in the basal diet as indicated in Tables I to III, an equivalent amount of glucose was omitted. All rats received orally 0.1 cc. of U. S. P. XI cod liver oil and 0.1 cc. of a solution containing 25  $\gamma$  of thiamine, 20  $\gamma$  of riboflavin, 100  $\gamma$  of calcium pantothenate, 100  $\gamma$  of nicotinic acid, and 20  $\gamma$  of pyridoxine per day. The experimental period was 21 days. The food intake was determined daily and weight changes were recorded three times weekly. The livers were removed from the animals under sodium amytal anesthesia and analyzed for total lipids (13). Apparent differences were analyzed for significance by the *t* method of Fisher (14) and only those showing a *P* value of less than 0.01 were considered significant.

### Results

Table I shows the pertinent data obtained when Diet 26 was supplemented with increasing quantities of methionine. It is evident that 500 mg. of methionine and 100 mg. of cystine per 100 gm. of the basal diet were insufficient to support optimum growth. Increasing the methionine to 600 mg. produced a highly significant increase in the growth rate. There was no additional stimulation of growth by the higher levels of Diets 28, 33, and 36. Under the present experimental conditions and in rats of this strain and age, approximately 40 per cent change in weight appears to be optimum. That optimum growth was obtained with 600 mg. of methionine rather than 500 mg., as found by Womack and Rose (8), is not necessarily in disagreement with their findings. In the present study the animals were older and the diets contained 40 per cent fat, whereas the diets used in their study contained 30 per cent. Moreover, it has been shown by duVigneaud and coworkers (15) that the vitamin B complex supplement used by Womack and Rose contained an appreciable quantity of choline, while our diets were choline-free.

<sup>1</sup> Generously supplied by the Corn Products Refining Company, New York.

<sup>2</sup> Proflo brand, kindly furnished by the Traders Oil Mill Company, Fort Worth.



Increasing the methionine from 500 to 600 mg. produced a slight but significant decrease in the liver lipids. The animals on Diet 27 ingested an average of 10.3 mg. of methionine per day more than those receiving Diet 26. This amount of methionine exhibited an effect on the growth rate and on the level of liver fat. The possibility of further differentiation between the growth and lipotropic requirements by using small increments of methionine between 500 and 600 mg. per 100 gm. of diet has not been investigated. Additional amounts of methionine produced further decreases until with Diet 36, which contained 1200 mg., the level of liver fat was essentially normal. The data indicate that in the absence of choline the total methionine requirement is approximately 1200 mg. per 100 gm. of diet and that the requirement for lipotropism is about 600 mg. In our

TABLE I

*Methionine Requirements for Growth and Lipotropism in Rats Receiving Choline-Free Diets*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 169 to 172 gm.

Diet No.	Methionine	Cystine	No. of rats	Food intake per day*	Gain in weight	Liver lipids per 100 gm.	
						Moist tissue	Body weight
	mg. per 100 gm. diet	mg. per 100 gm. diet		gm.	per cent	gm.	gm.
26	500	100	15	9.1 ± 0.3	23.8 ± 2.3	24.7 ± 0.9	1.45 ± 0.10
27	600	100	8	9.3 ± 0.3	37.0 ± 2.5	20.5 ± 1.0	0.97 ± 0.08
28	700	100	8	9.4 ± 0.3	38.3 ± 1.5	18.8 ± 1.5	0.89 ± 0.10
33	1000	100	8	10.0 ± 0.3	43.3 ± 2.0	14.9 ± 1.8	0.59 ± 0.08
36	1200	100	8	10.3 ± 0.1	41.8 ± 1.7	8.9 ± 0.3	0.32 ± 0.05

\* Including the standard error of the mean, calculated as follows:  $\sqrt{\Sigma d^2/n - 1}/\sqrt{n}$ .

previous work (4) with low protein diets the amount of fat in the liver was normal or only slightly increased when the diets contained approximately 600 mg. of methionine. Thus, in accord with the discussion in the introductory part, if the requirement for lipotropism is differentiated from the growth requirement, the amount of methionine used in lipotropic activity appears to be independent of the protein content of the diet.

The effect of adding various amounts of cystine to the basal diet is shown in Table II. There was no significant effect on the growth rate. In the present experiment it is impossible to evaluate the effect of the 100 mg. of cystine in the basal diet. However, the results are in agreement with the findings of Womack and Rose (8) that amounts greater than 100 mg. have no effect on the growth rate. There was no change in the liver lipids until

the cystine content was 600 mg. per 100 gm. of diet. At this level there was a distinct lipotropic effect. We have no explanation for the absence of any antilipotropic effect of the cystine in these diets. In this regard it may be significant that the antilipotropic action has usually been demonstrated with low protein-low methionine diets. We are not aware of any previous study in which cystine was shown to have a lipotropic effect. Experiments are being carried out to confirm and extend this observation.

TABLE II

*Cystine Requirements for Growth and Lipotropism in Rats Receiving Choline-Free Diets*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 170 to 173 gm.

Diet No.	Methionine	Cystine	No. of rats	Food intake per day	Gain in weight	Liver lipids per 100 gm.	
						Moist tissue	Body weight
	mg. per 100 gm. diet	mg. per 100 gm. diet		gm.	per cent	gm.	gm.
26	500	100	15	9.1 $\pm$ 0.3	23.8 $\pm$ 2.3	24.7 $\pm$ 0.9	1.45 $\pm$ 0.10
29	500	200	8	8.8 $\pm$ 0.2	26.4 $\pm$ 2.9	24.3 $\pm$ 0.8	1.47 $\pm$ 0.09
30	500	300	8	8.8 $\pm$ 0.1	29.3 $\pm$ 3.0	26.1 $\pm$ 0.6	1.71 $\pm$ 0.08
37	500	400	8	9.9 $\pm$ 0.4	30.6 $\pm$ 4.7	21.9 $\pm$ 1.8	1.30 $\pm$ 0.11
38	500	600	7	10.2 $\pm$ 0.3	26.5 $\pm$ 2.3	16.1 $\pm$ 1.0	0.99 $\pm$ 0.08

TABLE III

*Choline Requirements for Growth and Lipotropism*

The animals received the diets for 21 days. The average initial weights for the dietary groups ranged from 170 to 173 gm.

Diet No.	Methionine	Cystine	Choline	No. of rats	Food intake per day	Gain in weight	Liver lipids per 100 gm.	
							Moist tissue	Body weight
	mg. per 100 gm. diet	mg. per 100 gm. diet	mg. per 100 gm. diet		gm.	per cent	gm.	gm.
26	500	100	0	15	9.1 $\pm$ 0.3	23.8 $\pm$ 2.3	24.7 $\pm$ 0.9	1.45 $\pm$ 0.10
34	500	100	100	8	8.6 $\pm$ 0.2	20.2 $\pm$ 1.8	7.0 $\pm$ 0.8	0.27 $\pm$ 0.06
35	500	100	200	14	9.8 $\pm$ 0.3	27.2 $\pm$ 1.8	6.6 $\pm$ 0.3	0.23 $\pm$ 0.01

The data obtained when the basal diet was supplemented with choline are summarized in Table III. The growth rate was not influenced by the choline. It is interesting that while it has been reported that choline has a growth-stimulating effect (16, 17) none was found in this study. It would appear that conditions were favorable to show such an action of choline, inasmuch as the basal diet was choline-free and growth was less than optimum. In another study,<sup>3</sup> we have not observed a growth-promoting

<sup>3</sup> Tidwell, H. C., and Treadwell, C. R., unpublished data.

effect of choline. Apparently only under special conditions can this action be demonstrated. The supplementary choline exhibited its usual effect on the level of the liver lipids. 100 mg. per 100 gm. of diet lowered the level to normal. The average daily intake per rat was 8.6 mg. and the data do not exclude the possibility that still smaller amounts would give a maximum effect. This is the same order of activity as that found by Channon *et al.* (18).

The results of the present study suggest that the methionine requirement of the white rat is not a unitary one but is composed of two components. When the diet contains optimum quantities of the other essential food factors, a certain amount of methionine is needed for maximum utilization of the food in growth and maintenance; this amount is termed the growth requirement. Concurrent with optimum growth there may occur an abnormal accumulation of fat in the liver due to an inadequate intake of lipotropic factors. The organism's need for lipotropic factors may be met by choline, methionine, betaine, and other substances capable of donating methyl groups. If sufficient choline or related substances are present in the diet, no methionine will be required for lipotropism. However, if the diet is free of other lipotropic factors, then the methionine needed for lipotropic action becomes an important part of the total methionine requirement.

#### SUMMARY

Under the conditions obtaining in this study, the total methionine requirement of rats receiving a choline-free diet was approximately 1200 mg. per 100 gm. of diet. This total was differentiated into a growth requirement of 600 mg. and a lipotropic requirement of 600 mg.

Amounts of cystine greater than 100 mg. per 100 gm. of diet did not exhibit any effect on the growth rate or any antilipotropic activity. 600 mg. of cystine excited a slight lipotropic effect.

When the basal diet was supplemented with 100 or 200 mg. of choline per 100 gm. of diet, there was no increase in the growth rate. The 100 mg. level gave a maximum lowering of liver fat.

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## THE DETERMINATION OF ASCORBIC ACID IN SMALL AMOUNTS OF BLOOD SERUM

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The measurement of the ascorbic acid level in serum has attained considerable importance as an index of ascorbic acid nutrition. Existing ascorbic acid methods require at least 0.1 ml. of blood serum or 0.2 ml. of blood (1, 2), an amount which can just be obtained from the finger. If, as is usually the case in making nutritional surveys, it is desired to measure a number of other blood constituents, resort must be had to venipuncture to obtain sufficient blood.

The advantage of avoiding venipuncture in dealing with large groups, especially when children are concerned, has led to an exploration of the possibility of determining ascorbic acid on much less than 0.1 ml. of serum.

Three different reagents have been used successfully for measuring ascorbic acid in blood serum: dichlorophenol indophenol (Mindlin and Butler (1)), methylene blue (Butler, Cushman, and MacLachlan (2)), and dinitrophenylhydrazine (Roe and Kuether (3)). The first two reagents measure ascorbic acid directly, whereas dinitrophenylhydrazine measures ascorbic acid only after oxidation to dehydroascorbic acid. All three reagents have been investigated for their adaptability to small scale analyses.

Of the three, the methylene blue reagent is by far the most sensitive, but its use requires an irradiation step which is somewhat awkward in dealing with small volumes. With dichlorophenol indophenol it was found possible to measure the ascorbic acid in 0.01 ml. of serum, but it proved difficult when handling large numbers of determinations to avoid some prior loss of ascorbic acid through oxidation. Therefore, the choice fell upon dinitrophenylhydrazine. Obviously, with this reagent prior oxidation of ascorbic acid to dehydroascorbic acid, if it should occur, would do no harm, since in any event all of the ascorbic acid must finally be converted to dehydroascorbic acid before measurement. It has been found that dehydroascorbic acid (or a derivative which reacts with dinitrophenylhydrazine (4)) is remarkably stable in serum after trichloroacetic acid addition, whether or not the extract is separated from the protein precipitate. This has proved of great advantage in carrying out large numbers of analyses under conditions of a nutritional survey.

It has proved quite simple to adapt the Roe and Kuether dinitrophenylhydrazine method to the measurement of the ascorbic acid in 0.01 ml. of

serum. With the resulting procedure, one person can analyze 50 sera per day. In adapting the procedure to a small scale, it was necessary to make certain simplifications which should prove helpful in large scale work as well.

### *Method*

#### *Reagents and Equipment—*

1. 2 per cent dinitrophenylhydrazine, 0.25 per cent thiourea, in 9 N  $\text{H}_2\text{SO}_4$ ; centrifuge or filter through sintered glass if a precipitate develops. Add the thiourea only to sufficient reagent for 1 month's use, store in the ice box, and discard the remainder of this portion after 1 month.

2. 65 per cent  $\text{H}_2\text{SO}_4$  prepared by adding 70 ml. of concentrated  $\text{H}_2\text{SO}_4$  to 30 ml. of  $\text{H}_2\text{O}$ .

3. 1 per cent suspension of norit in 5 per cent trichloroacetic acid. The norit is washed with acid and dried according to Roe and Kuether (3), and 5 gm. are suspended in 100 ml. of 5 per cent trichloroacetic acid. After settling, the supernatant is decanted and the volume restored with 5 per cent trichloroacetic acid. This is repeated several times to eliminate some of the excessively fine, floating charcoal. Once a week or so the supernatant acid from a small portion is replaced with fresh acid to eliminate the possibility of contamination with heavy metals which, according to Roe and Kuether, may slowly leach out of the norit.<sup>1</sup>

4. Levy-Lang constriction micro pipettes (hand type), 10, 30, and 50 c.mm. (5). In addition, for pipetting the charcoal suspension, a 40 c.mm. pipette with tip and constriction 2 or 3 times wider than normal to avoid plugging with charcoal.

5. A Beckman spectrophotometer fitted with a special diaphragm and cuvettes to permit the use of 0.05 ml. fluid volumes<sup>2</sup> (obtainable from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28).

6. 6  $\times$  50 mm. serological tubes, *e.g.* Kimble, No. 45060.

*Procedure* To the bottom of a 6  $\times$  50 mm. tube are transferred 10 c.mm. of serum<sup>3</sup> and 40 c.mm. of the acid-charcoal suspension (Reagent 3), and the contents mixed by tapping the tube with the finger. (The charcoal suspension in a small vessel is stirred immediately before each pipetting by blowing through the pipette, and the pipette must be filled and emptied rapidly to avoid plugging due to settling of the charcoal.) The tube is capped with a piece of parafilm or a rubber stopper and centrifuged 10

<sup>1</sup> If charcoal gets into the final sample, low values may result. If difficulty is encountered from floating charcoal, 1 volume of 2 per cent gelatin may be added to 10 volumes of the acid-charcoal suspension just before use.

<sup>2</sup> Lowry, O. H., and Bessey, O. A., in preparation.

<sup>3</sup> A convenient method of collecting small amounts of blood for this purpose from the finger has been described (6).

minutes at 3000 R.P.M. A 30 c.mm. aliquot of the supernatant is transferred to another 6 × 50 mm. tube, and 10 c.mm. of the thiourea-dinitrophenylhydrazine reagent (Reagent 1) are added with tapping. The tube is capped as before and incubated for 3 hours at 38°. The tube is now chilled in ice water and 50 c.mm. of ice-cold 65 per cent H<sub>2</sub>SO<sub>4</sub> are added, *very thoroughly mixed*, and read after 30 minutes at room temperature in the spectrophotometer at a wave-length of 520 mμ. The sample is transferred to the spectrophotometer with a short Pasteur pipette. Standards and blanks are provided by adding 4 ml. of the acid-charcoal suspension to 1 ml. aliquots of fresh 1 mg. per cent ascorbic acid solution and water, respectively. After centrifuging, 30 c.mm. aliquots are treated in the same manner as the unknowns. Care is taken to avoid floating charcoal, which is more troublesome in the absence of serum. After correction for the blank, the color produced is directly proportional to the concentration of ascorbic acid and, therefore, only the 1 mg. per cent standard is required for routine purposes. In practice, a long series of samples is placed in order in a metal rack and carried through the analysis together.

Once acid has been added to the serum, it may be safely stored several days in the ice box or for several weeks at -20°. If the supernatant acid extract is separated, it may be stored without loss of the ascorbic acid or its derivatives for at least several weeks in the ice box, and presumably indefinitely at -20° (see below). The chief consideration is to prevent evaporation of the small samples. Rubber stoppers have proved most effective. The cut off smaller ends of No. A vial stoppers have been found very convenient for the 6 × 50 mm. tubes. Parafilm is not reliable for the prevention of evaporation from these tubes for more than a short time.

#### *Adaptation to 25 C.mm. of Serum*

##### *Apparatus--*

1. Constriction pipettes, 25, 75, and 125 c.mm.; also a 100 c.mm. pipette with wider constrictions for pipetting the charcoal suspension.

2. Either the Beckman spectrophotometer or the Coleman Junior spectrophotometer (model 6) with cells and adapter for 0.2 ml. volume. (The adapter is obtainable from Samuel Ash, 3044 Third Avenue, New York 56.)

3. Reagents and tubes as described above.

The procedure is identical to that described above except that all volumes are increased proportionately. To 25 c.mm. of serum in a 6 × 50 mm. tube are added 100 c.mm. of the acid-charcoal suspension. After centrifuging, a 75 c.mm. aliquot is transferred to another 6 × 50 mm. tube and 25 c.mm. of the diphenylhydrazine reagent (No. 3) are added. After incubation 125 c.mm. of 70 per cent sulfuric acid are added.

Adaptation to still larger serum volumes may be effected by simple



multiplication of all volumes, with a not inconsiderable increase in convenience as compared to the original Roe and Kuether procedure.

#### DISCUSSION

Aside from requiring smaller quantities throughout, the deviations from the Roe and Kuether procedure consist of (a) a reduction in the relative quantity of charcoal employed; (b) the addition of the charcoal as a suspension in trichloroacetic acid instead of as a dry powder; (c) the removal of protein and charcoal in one step by centrifuging instead of first centrifuging the protein, then adding charcoal and filtering; (d) combination of the thiourea and dinitrophenylhydrazine reagents; and (e) reduction in the strength of the strong sulfuric acid employed.

These changes reduce the number of operations and add greatly to the convenience of the procedure on a small scale. Filtration is scarcely practicable with volumes of the order of 0.05 ml. Although the bulk of the charcoal is easily centrifuged, there is a tendency for some of it to float. This tendency was decreased (so that upon the addition of serum the floating was entirely eliminated) by suspending the charcoal and decanting the floating fraction, and by using less charcoal, since a smaller amount could be shown to suffice for complete conversion of ascorbic acid to dehydroascorbic acid. Removal of protein and ascorbic acid in one step not only reduced the number of manipulations required, but also tended to carry down the charcoal more completely. It was found that identical results were obtained whether the charcoal was added to the serum before the trichloroacetic acid, after the trichloroacetic acid, or separately to the deproteinized serum supernatant. By reducing the strength of the sulfuric acid to be added after incubation from 85 to 65 per cent and chilling this sulfuric acid, the heat produced on mixing was so decreased that the acid could be added all at once, whereas by the original procedure it was necessary to add the sulfuric acid slowly over the course of a minute to prevent charring. A slow addition of this type would be awkward with the small volumes employed here. To compensate for this decrease in acid concentration, proportionately more was used.

Certain analytical considerations which were thought to be of possible significance have been checked and shown to be without influence on the results. These are (a) the age of the trichloroacetic acid; (b) the size and quality of the glass tubes, whether soft glass or Pyrex; (c) the separate addition of the trichloroacetic acid and charcoal in water suspension in any order as compared to addition of the two together; (d) the age of the trichloroacetic-charcoal reagent; and (e) variation in charcoal concentration from 0.2 to 4 per cent (but with less charcoal there is less tendency for the charcoal to float).

On the other hand, a significant increase in thiourea decreases color development, whereas omission of thiourea increases the blank reading. Decreasing the final acid strength below that recommended decreases the amount of color development.

*Comparative Serum Analysis*—In Table I are recorded the results of a comparison between the proposed method in which 0.01 ml. of serum is used and the Mindlin and Butler dichlorophenol indophenol method with 1 ml. of serum. The Roe and Kuether micromethod was applied both to individual serum samples obtained from finger blood centrifuged in capillary tubes (6) and to aliquots of a large serum sample obtained by vein. The correlation appears to be quite satisfactory between the two

TABLE I  
*Comparative Ascorbic Acid Analyses of Blood Serum*

The values are given in mg. per cent.

Subject No.	Dichlorophenol indophenol (macro)	Roe and Kuether, vein (micro)	Roe and Kuether, finger (micro)	Bromine,* vein (micro)	Bromine minus Roe and Kuether, vein
1		1.92	1.84	1.97	+0.05
2	1.31	1.34	1.34	1.52	+0.18
3	1.33	1.33	1.35	1.40	+0.07
4	1.01	0.93	1.03	1.18	+0.25
5	1.14	1.11	1.23	1.24	+0.13
6	0.24	0.13	0.20		
7	1.10	1.05	1.12	1.12	+0.07
8	0.32	0.27	0.28	0.28	+0.01
9	1.64	1.63	1.65	1.65	+0.02
10	1.26	1.21	1.28	1.28	+0.07
Average.....					+0.12

\* 1 c.mm. of 2.5 per cent bromine in water.

methods and also between the small finger blood samples and the large venous samples. The values recorded for the small samples are the average of triplicate determinations.

An attempt was made to substitute bromine for charcoal (as the oxidant for ascorbic acid). 1 c.mm. of 2.5 per cent bromine water was added to each tube in place of the charcoal. The procedure worked smoothly with pure ascorbic acid samples, but as is seen in the last column of Table I, there was with serum a tendency for bromine to produce slightly higher results than the other procedures. Evidently charcoal, in addition to oxidizing the ascorbic acid, removes some interfering material. Nevertheless, it may be that for some purposes the bromine procedure would be advantageous, since the use of the somewhat troublesome charcoal reagent

would be avoided. In this case, the thiourea must be added as a separate solution before the rest of the reagent.

*Proportionality and Reproducibility*—The color development was found to be satisfactorily proportional on the micro scale. For example, with standard solutions equivalent to 0.2, 0.4, 0.8, 1.6, and 3.2 mg. per cent of serum, observed density readings were 0.013, 0.034, 0.069, 0.133, and 0.276, whereas the readings, if proportional, should have been 0.017, 0.034, 0.069, 0.138, and 0.276. Replicate determinations agree satisfactorily. For a series of forty-six sera ranging from 0.3 to 1.4 mg. per cent, analyzed in duplicate, the standard deviation of the individual determination was 0.03 mg. per cent.

TABLE II

*Effect of Storage on Ascorbic Acid Values of Blood Serum (Ascorbic Acid Plus Dehydroascorbic Acid)*

Per cent ascorbic acid remaining									
Serum (hemo- lyzed)*	Serum						Serum + acid		
	38°		4°		-20°		4°	-20°	
	1 hr.	4 hrs.	6 days	13 days	6 days	13 days	13 days	13 days	13 days
62	95	55	41	6	90	57	96	95	

Serum + acid + norit					Acid extract				
25°		4°	-20°		25°	4°		-20°	
2 days	8 days	2 days	2 days	8 days	6 days	6 days	13 days	6 days	13 days
101	145	104	105	102	95†	103†	100	100†	100

\* Serum with 2 per cent hemolyzed blood added.

† Stored with 3 per cent metaphosphoric acid. All the rest stored with 4.5 per cent trichloroacetic acid.

*Stability*—The advantage of the dehydroascorbic acid method from the standpoint of stability is shown by the results of storage tests at various temperatures on serum, serum with added trichloroacetic acid (Table II), serum with added norit and trichloroacetic acid, and extracts of serum made with norit and trichloroacetic acid or metaphosphoric acid. Serum was stored in capillary tubes. For the other samples, 10 c.mm. serum aliquots in 6 × 50 mm. tubes were precipitated with 30 c.mm. of acid with or without added norit and were either stored in these tubes without separating the supernatant or the supernatant was transferred to other 6 × 50 mm. tubes for storage. The samples were analyzed at various times up to 13 days. It is evident that no loss was detectable even at room

temperature with the acidified samples, and even the serum stored at 4° contained considerable amounts of ascorbic acid (presumably as dehydroascorbic acid) after 6 days. Under these conditions only the frozen samples would have contained detectable amounts of reduced ascorbic acid after 2 days; *i.e.*, all but possibly the frozen samples would have given zero values with dichlorophenol indophenol. However, in spite of this considerable stability, some care must be taken in hot weather to keep blood or serum samples cool until they are acidified, since at 38° there is detectable loss in 1 hour and serious loss in 4 hours (Table II). It is further indicated that hemolysis tends to accelerate the process of destruction; hence special care should be taken to keep hemolyzed specimens cool.

It will be noted that for samples stored with norit at room temperature the absorption values increased; evidently the charcoal caused the formation of something which reacts with the Roe and Kuether reagent. The tendency for the charcoal to float is somewhat increased by freezing and the subsequent necessary stirring. This makes it advisable, when samples are to be stored in the frozen state, to separate the supernatant before freezing.

The advantage of being able to store acidified samples until a large number have been collected is obvious. Because of this and the other advantages ascribed to it, the proposed method appears to satisfy the requirements of a micromethod for purposes of nutritional surveys.

#### SUMMARY

The dinitrophenylhydrazine method of Roe and Kuether has been adapted to the determination of ascorbic acid in 0.01 ml. of serum. The values obtained are in agreement with those obtained by the macro indophenol method of Mindlin and Butler.

Necessary changes in procedure for adaptation to microanalyses appear to represent simplifications applicable to macroanalyses as well.

The micromethod is particularly suited for nutritional surveys owing to (a) the ease of obtaining suitable blood specimens, (b) the stability of specimens, and (c) the convenience for large scale analytical operations.

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# PHOTOMETRIC DETERMINATION OF POTASSIUM IN BIOLOGICAL MATERIALS

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In the course of work in this laboratory (1-2) the need of simple, sensitive, and reasonably accurate methods was felt for the determination of several biologically important ions on small quantities of material. The present paper reports our work on the determination of potassium.

The colorimetric methods for potassium are all indirect and require the isolation of the element as some slightly soluble compound containing a constituent capable of giving an intensely colored product with a suitable reagent. Potassium is determined colorimetrically by first isolating it as the potassium chloroplatinate, potassium, sodium, or silver cobaltinitrite, or potassium dipicrylamine. The chloroplatinate method is the oldest and generally considered to be the best for determining macro and micro quantities of potassium, but on account of the high price of platinum, and the consequent necessity of recovering the platinum from the residues, less expensive procedures have been sought. The dipicrylamine reagent has been recently introduced into quantitative analysis (3-5) and may be applied to the determination of 0.01 to 0.1 mg. of potassium. Sodium cobaltinitrite is probably the most sensitive precipitant of potassium (6), and in halogen-free solutions an even more insoluble precipitate is obtained by using silver cobaltinitrite as a precipitant (7-8). The difficult point to observe with these reagents is the structure of the precipitate which very often has been questioned, and formulae ranging from  $\text{KNa}_2(\text{Co}(\text{NO}_2)_6)$  to  $\text{K}_2\text{Na}(\text{Co}(\text{NO}_2)_6)$  have been reported, depending on the conditions under which the precipitation is carried out (9). Hubbard (10) found that the relative concentrations of potassium, sodium, cobalt, and nitrite ions and the acidity of the solution were the factors which most markedly affected the composition of the precipitate, and recommends adding sodium acetate to obtain approximately theoretical results. Jendrassik (11-12) found that high precision is attained only if potassium is precipitated by adding separated solutions of cobalt nitrate and sodium nitrite. Sobel and Kramer (13) found that under the conditions of their technique the constant atomic ratio of K:Co was 2:1.2. Robinson and Putnam (14), by using a modification of the method of Breh and Gaebler (15) and applying it to the determination of potassium in water, obtained a precipitate of definite structure,

the composition of which was best represented by the formula  $K_{1.35}Ag_{1.65}(Co(NO_2)_6)$ . Robertson and Webb (16) used the silver cobaltinitrite reagent for estimating potassium in sea water and blood and in body fluids of marine animals.

The variations reported have led to the conviction that the cobaltinitrite method is less accurate because of the variable K:Na ratio in the precipitate; so that it is generally agreed that the composition of the precipitate is best represented by the formula  $(KNa)_3(Co(NO_2)_6)$  which expresses well the property which sodium and potassium have of forming a series of double nitrites with cobalt. Nevertheless, under regulated conditions of precipitation the composition is fairly reproducible and many chemists have obtained excellent results with the cobaltinitrite method.

To determine indirectly the potassium, the diazotization of an aromatic amine with the nitrite content of the precipitate followed by a coupling reaction has been extensively used (12, 17-22). It seems, however, that the more stable cobalt is to be preferred. Tomula (23) develops the blue color with thiocyanate in 75 per cent acetone; Durupt and Schlesinger (24), also Gerschmann and Marenzi (25), employ Vogel's reaction, but the blue color is extracted with ether-amyl alcohol mixture. Yoshimatsu (26) dissolves the sodium potassium cobaltinitrite precipitate in nitric acid and compares the color given by cobalt with dimethylglyoxime and sodium sulfide. Jacobs and Hoffman (27), after dissolving the precipitate in hot water, add choline hydrochloride and potassium ferrocyanide; an emerald-green color develops. Sobel and Kramer (13) dissolve the potassium sodium cobaltinitrite in hydrochloric acid, evaporate to dryness, dissolve the residue in a potassium pyrophosphate solution, and add a solution of cysteine hydrochloride and hydrogen peroxide; an intense yellow color develops which is compared in a colorimeter against a standard made simultaneously.

Breh and Gaebler (15) determine indirectly the potassium in the potassium silver cobaltinitrite by employing the reaction between cobalt and ammonium thiocyanate in alcoholic solution and use a curve to correct for the discrepancy between color intensity and cobalt concentration. Robinson and Putnam (14) determine the nitrite content of the potassium silver cobaltinitrite by the extremely sensitive Griess method.

Harris (28) in his modified silver cobaltinitrite method for potassium determines the nitrite contents of the precipitate, as did Robinson and Putnam.

In the method to be described in the present paper, potassium is isolated as the potassium silver cobaltinitrite and cobalt is determined photometrically after adding dimethylglyoxime and benzidine. The qualitative color reaction was described by Chiarottino (29) and found to be highly sensitive

by Spacu and Macarovici (30) who applied it to the colorimetric determination of cobalt by comparing in a colorimeter the color intensity of the sample against a standard prepared simultaneously.

### *Method*

#### *Reagents---*

*A. Silver cobaltinitrite.* This reagent is prepared according to the directions given by Robinson and Putnam (14). Dissolve 25 gm. of sodium cobaltinitrite in 150 ml. of sodium nitrite solution containing 50 gm. of sodium nitrite. Add with stirring 5 ml. of a 40 per cent solution of silver nitrate, dilute to 200 ml. with distilled water, add 2 ml. of glacial acetic acid, and draw air through the cold solution until the evolution of gas has passed off. The reagent is placed in the ice chest and after standing for 12 to 24 hours at 4-6° is filtered by gentle suction on a fine Jena glass filter and preserved in a dark glass-stoppered bottle in the ice box at 4-6° when not in use. Just before use a portion is filtered as above. Reacerate weekly and discard after 1 month.

*B. Washing liquids.* Distilled water; 50 per cent acetone; pure acetone. The acetone must give an entirely negative test with silver nitrate.

*C. Hydrochloric acid,* sp. gr. 1.19.

*D. Dimethylglyoxime reagent.* 1 gm. of dimethylglyoxime in 100 ml. of 95 per cent ethyl alcohol.

*E. Benzidine reagent.* 1 gm. of benzidine in 100 ml. of 95 per cent ethyl alcohol. Keep in a dark glass-stoppered bottle away from sunlight; discard if color develops. All of the chemical and solutions must be of the purest grade, as free from impurities as possible.

### *Procedure*

Pyrex conical bottomed centrifuge tubes graduated at 5, 7.5, and 10 ml. are used in our technique. An exactly measured quantity of the ammonium- and halogen-free, neutral or slightly acid (acetic acid) solution to be tested is introduced into the centrifuge tube and 1 ml. of Reagent A is added to each ml. of the potassium-containing solution. Mixing is effected by holding the tube between the thumb and the forefinger of one hand and flipping the fingers of the other hand rapidly against the bottom of the tube. After standing in the ice box at 4-6° for at least 2 hours, the precipitate and solution are centrifuged for about 15 minutes at 3000 R.P.M.

The supernatant liquid is drawn off to within about 0.5 ml. by means of a capillary with an upturned tip so as not to disturb the precipitate. The first washing is made with distilled water. Add about 1 ml. of water, drop by drop, letting the liquid run along the walls of the tube so as not to raise the precipitate. In this way two liquid layers are formed, one the almost undiluted silver cobaltinitrite reagent, the other a very dilute solution of



the same reagent. In order to mix these layers with as little disturbance of the precipitate as possible, carefully incline the tube to an almost horizontal position and rapidly turn it up. After repeating these movements three or four times, wash the entire inside of the tube with 4 ml. of distilled water added from a pipette moved around the top of the tube. Centrifuge for about 10 minutes at 3000 R.P.M. and withdraw the supernatant liquid as before. Repeat the washing and withdrawal, using 50 per cent acetone instead of distilled water. Next wash with pure acetone. After centrifuging for about 5 minutes at 3000 R.P.M., if care is taken to "bed" the precipitate down well, the liquid can be poured off without any loss of the precipitate. Carefully decant the supernatant liquid, drain well on filter paper, and wipe off the edge of the tube with a clean cloth or lintless filter paper. Wash once again with pure acetone, centrifuge, decant, drain well, and wipe off the edge of the tube as before. The supernatant liquid should be colorless at the end of the second washing with pure acetone. If not, wash again with acetone. Next add 0.1 ml. of hydrochloric acid, sp. gr. 1.19, and evaporate to dryness on an air bath, the temperature of which is slowly raised to about 150–160°. The air bath is covered by a perforated brass plate which holds the centrifuge tubes vertical.

After removing all excess acid, cool to room temperature, add distilled water, heat in a boiling water bath, cool again, add Reagent D, and afterwards Reagent E, and dilute the contents of the tube with distilled water to an appropriate volume and mix thoroughly.

The photometric measurements are made with the Zeiss Pulfrich step-photometer. The technique employed in making the measurements is that described in the directions which accompany the instrument.<sup>1</sup> The extinction,  $E$ , was either read from a calibrated drum on the photometer or calculated from the transparency,  $D$ . The extinction coefficient,  $k$ , was then obtained by dividing  $E$  by  $s$ , the length of the cell used;  $k = E/s$ .

#### *Photometric Determination of Cobalt*

In order to apply Chiarottino's test reaction to the indirect determination of potassium isolated as the potassium silver cobaltinitrite, a study was made on the photometric determination of cobalt by means of this color reaction which is very sensitive to small quantities of cobalt and useful for wide ranges of this element. The present author has found that this color reaction is suitable for the photometric determination of cobalt in the potassium silver cobaltinitrite precipitate.

*Procedure*—To the neutral solution of cobalt add Reagent D, and afterwards Reagent E, mix, dilute with distilled water to an appropriate volume, and mix thoroughly. Determine the extinction with the use of Filter S-43

<sup>1</sup> Mess 430 d/IV.

and cells of appropriate length. Such a depth of cell is selected that the extinction,  $E$ , lies between 0.15 and 0.80.

*Effect of Excess of Reagent on Color*—An excess of dimethylglyoxime has no effect on the transmittance. A cobalt solution, 1.45 mg. per 100 ml., was treated with 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.5, and 2.0 ml. of Reagent D, 0.2 ml. of Reagent E being added in each case. The values of  $k$  were as follows (0.25 cm. cell length): 1.948, 1.948, 1.960, 1.936, 1.948, 1.960, 1.936, and 1.948. The same does not hold for Reagent E. The above solution of cobalt treated with 0.5 ml. of Reagent D in each case and varying quantities of Reagent E, from 0.2 to 2 ml., gave extinction coefficients,  $k$ , which varied from 1.948 for 0.2 ml. of reagent to 2.176 when 2 ml. were added. From the data thus obtained it was concluded that Reagent E must be used discreetly, and that unnecessarily large amounts of it are not to be added to the sample to be analyzed. 0.2 ml. of Reagent E for 10 ml. final volume is the amount used in the present technique.

TABLE I  
*Effect of Final Volume on Color*

The solution contained 0.5 mg. per 100 ml. of Co in each case.

Final volume	Cell length	$E$	$k$
ml.	cm.		
2.5	0.5	0.337	0.674
5.0	1.0		0.670
7.5	1.0		0.670
10.0	1.0		0.670

*Stability of Color*—The maximum color develops within about 10 minutes after Reagents D and E are added, and remains unchanged for at least 20 hours if pure solutions of cobalt are used. This is shown in the following experiment.

A solution of cobalt, 0.50 mg. per 100 ml., was treated with 0.5 ml. of Reagent D and afterwards with 0.2 ml. of Reagent E. The values of  $k$  were as follows: 2 minutes 0.585, 3 minutes 0.602, 5 minutes 0.635, 10 minutes 0.658, 30 minutes 0.658, 3 hours 0.658, 20 hours 0.658.

*Effect of Final Volume on Color Readings*—A change of the final volume has no effect on the extinction, as is shown in Table I.

*Effect of Temperature on Color Readings*—It was found that anywhere in the range of 20–30°, which covers the usual room temperature, the color readings were identical.

*Lambert-Beer Law*—Under the conditions of the present technique the intensity of the color which develops is determined by the amount of cobalt present. Calibration graphs plotted with the per cent of cobalt and extinc-

tion coefficient  $k$  as coordinates were straight lines; an equation for the per cent of cobalt may thus be derived from the slope of the line.

The extinction coefficient,  $k$ , is the extinction for a 1 cm. layer of absorbing medium, and is directly proportional to the concentration of a solution, if the Lambert-Beer law holds. If so, the concentration of an unknown may be calculated simply by measuring the extinction of a layer of known thickness.

If  $k$  denotes the extinction coefficient of a solution of known concentration,  $c_1$ , the concentration of an unknown,  $c_2$ , giving an extinction coefficient,

TABLE II  
*Relation of Extinction Coefficient,  $k$ , to Concentration of Cobalt,  $c$*

Co	Cell length	$D$	$E$	$k$	$\frac{c}{k}$
<i>mg. per 100 ml.</i>	<i>cm.</i>				
0.050	3.0	63	0.201	0.067	0.746
0.100	3.0	39.4	0.405	0.135	0.741
0.150	3.0	25	0.602	0.201	0.746
0.200	2.0	29.4	0.532	0.266	0.752
0.250	2.0	21.4	0.670	0.335	0.746
0.300	1.0	39.6		0.402	0.746
0.350	1.0	34		0.469	0.746
0.400	1.0	29.4		0.532	0.752
0.450	1.0	25		0.602	0.748
0.500	1.0	21.4		0.670	0.746
0.600	1.0	16		0.796	0.751
0.700	0.5	34.4	0.463	0.926	0.756
0.800	0.5	29.4	0.532	1.064	0.752
0.900	0.5	25.4	0.595	1.190	0.756
1.000	0.5	21.4	0.670	1.340	0.746
Mean					0.749
" error of each determination, $\sqrt{\Sigma d^2/(n-1)}$					0.0445
" " " mean, $\sqrt{\Sigma d^2/(n(n-1))}$					0.0012

$k_2$ , is  $c_2 = (c_1/k_1) \times k_2$ ;  $k_2$  and  $c_2$  are variables and  $c_1/k_1$  is a constant which once determined will always hold for the method; so that the concentration of an unknown is readily calculated by multiplying the constant by the extinction coefficient.

Table II shows that the extinction coefficient,  $k$ , is directly proportional to the concentration of cobalt,  $c$ .

#### *Photometric Determination of Potassium*

*K:Co Ratio in Potassium Silver Cobaltinitrite; Calibration Factor for Potassium*—Potassium sulfate "*pro analysi*," Schering-Kahlbaum, was

weighed out accurately, dissolved in distilled water in a 1.000 ml. volumetric flask, and diluted to the mark with distilled water. Aliquots were taken and potassium was determined gravimetrically by the method accepted by the Association of Official Agricultural Chemists (31). Aliquots of this solution were then conveniently diluted. Potassium was precipitated as the potassium silver cobaltinitrite, the precipitate was washed as described, and cobalt determined photometrically by the above procedure. According

TABLE III  
*K:Co Ratio in Potassium Silver Cobaltinitrite Precipitate; Calibration  
Factor for Potassium*

Volume of colored solution, 10 ml. in each case.

K	Cell length	D	E	k	Co found	$\frac{C}{k}$
<i>mg. per 100 ml. colored solution</i>	<i>cm</i>				<i>mg. per 100 ml. colored solution</i>	
0.05	3.0	60	0.222	0.074	0.0554	0.676
0.08	3.0	43.5	0.362	0.121	0.0906	0.670
0.10	3.0	36.2	0.441	0.147	0.1101	0.680
0.15	3.0	21.6	0.666	0.222	0.1663	0.676
0.20	2.0	25.6	0.592	0.296	0.2217	0.676
0.25	1.0	42.5		0.372	0.2786	0.672
0.30	1.0	35.8		0.446	0.3341	0.673
0.35	1.0	30		0.532	0.3917	0.669
0.40	1.0	25.6		0.592	0.4434	0.676
0.45	1.0	21.6		0.666	0.4988	0.676
0.50	1.0	18		0.745	0.5580	0.671
0.60	0.5	36	0.444	0.888	0.6651	0.676
0.70	0.5	30	0.522	1.044	0.7820	0.670
0.80	0.5	25.6	0.592	1.184	0.8866	0.676
0.90	0.5	21.4	0.670	1.340	1.0037	0.672
1.00	0.5	18.2	0.740	1.480	1.1085	0.676
Mean						0.674
" error of each determination						0.003
" " mean						0.0008

to the results shown in Table III, if we assume that the potassium was completely precipitated, the formula of the potassium silver cobaltinitrite obtained under the above conditions may be represented as  $K_{1.36}Ag_{1.61}(Co(NO_2)_6)$ .

*Effect of Acidity on Precipitation of Potassium*—The solution of potassium to be tested must be neutral or slightly acid. We have always employed acetic acid and found that up to an acidity corresponding to 2 N no effect is seen on the precipitation of potassium. Potassium was determined on

aliquots of the same solution, according to the above procedure, under exactly the same conditions except for the quantity of acetic acid added, which was varied to give normalities ranging from 0.0, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, to 2. The values of  $k$  were as follows: 0.500, 0.495, 0.517, 0.490, 0.495, 0.509, 0.500, 0.495, and 0.495.

*Effect of Time on Precipitation*—1 ml. of a solution containing 30.6  $\gamma$  of potassium per ml. was treated with 1 ml. of Reagent A and allowed to stand in the ice box at 4–6° for various periods ranging from 30 minutes to 18 hours. Potassium was then determined as described in the procedure, the volume of the colored solution being 10 ml. in each case. The solutions showed the following extinction coefficients,  $k$ , after various times of standing: 30 minutes 0.398, 1 hour 0.420, 2 hours 0.456, 3 hours 0.449, 18 hours 0.449. From these data it was concluded that 2 hours standing in the ice chest at 4–6° is long enough to secure maximum precipitation of potassium under the conditions of the procedure, and that longer times, at least up to 18 hours, are not prejudicial.

*Stability of Color*—As has already been shown, if pure solutions of cobalt ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) are used, the color remains unchanged for at least 20 hours. The same does not hold for the color developed after the potassium silver cobaltinitrite complex is destroyed with hydrochloric acid and the resulting solution evaporated to dryness, as is shown in the following experiments. A solution of potassium, 0.50 mg. per 100 ml., was treated according to the conditions prescribed. The colored solution showed the following values of  $k$  after various times of standing: 2 minutes 0.638, 5 minutes 0.674, 10 minutes 0.734, 15 minutes 0.734, 30 minutes 0.734, 45 minutes 0.734, 1 hour 0.754, 28 hours 0.886. This is probably due to the catalytic action of silver chloride present in the system.

#### *Determination of Potassium in Biological Materials*

*Ashing*—We have used wet ashing to remove ammonium and halogen ions from the incinerated material. Ammonium salts give a precipitate similar to that produced by potassium and must therefore be completely eliminated before precipitating potassium, and obviously the solution must be halogen-free. Halogens could be precipitated with a silver nitrate solution but, as Robinson and Putnam found, only a slight excess of silver ion should be present from the precipitation of the chloride, for, otherwise, a higher silver-potassium ratio in the precipitate is to be noted. In view of this, we have tried to avoid precipitating the halogens existing in the solution to be tested by means of silver nitrate. To this effect the following modification was introduced in the wet ashing procedure previously used (32).

A sample of convenient size is accurately measured and placed in a 180  $\times$

15 mm. Pyrex, or preferably fused silica, test-tube graduated at 1 ml. 0.5 ml. of a nitric-perchloric acid mixture (3 ml. of  $\text{HNO}_3$ , sp. gr. 1.40, and 1 ml. of  $\text{HClO}_4$ , sp. gr. 1.67) is added and the tube is heated on an air bath, the temperature of which is slowly raised to about  $135^\circ$ . The air bath is covered by a perforated brass plate which holds the tube vertical. The acid solution boils gently. After the brown fumes of oxides of nitrogen have evolved, if charring occurs, add measured quantities of the acid mixture (about 0.2 ml. at a time) and continue until a colorless or very pale yellow liquid is obtained. The temperature of the air bath is then slowly raised to about  $205^\circ$ , until white fumes appear. If the solution is not colorless at this point, add more of the acid mixture as before, and heat at about  $135^\circ$  and then at about  $205^\circ$ . After a white, or very pale yellow, residue is obtained, raise the temperature of the air bath to about  $250^\circ$ , until all excess of perchloric acid has been removed. The test-tube is then heated more vigorously on a micro burner flame. According to the amount of nitrogen present in the sample, and thus to the quantity of ammonium perchlorate formed in the process, very slight explosions may be observed in the tube. The heating is continued until all fumes have evolved. This eliminates ammonium. Cool the tube, add 0.5 ml. of 2 N  $\text{H}_2\text{SO}_4$ , evaporate on the air bath at about  $130^\circ$ , and then heat on a small flame, with continuous shaking, until white fumes appear. Continue the heating until there is free evaporation of the sulfuric acid. Cool, add 0.5 ml. of distilled water, heat the test-tube slightly, and then cool. Dilute exactly to the mark. Run a standard at the same time, under exactly the same conditions. The acid solution obtained at the end of the process is ammonium- and halogen-free.

*Determination of Potassium in the Ash*—A convenient aliquot is taken from the cold acid solution of the ash and transferred to a Pyrex conical bottomed centrifuge tube graduated at 5, 7.5, and 10 ml. Add a drop of methyl red, and afterwards, drop by drop, a concentrated solution of sodium carbonate until the solution becomes alkaline. Plunge the tube in a boiling water bath for about 5 minutes. If a precipitate, or turbidity, forms at this stage, cool, centrifuge, and decant the supernatant liquid to a similar Pyrex centrifuge tube. Dissolve the precipitate in a few drops of 2 N  $\text{CH}_3\text{COOH}$ , add about 0.5 ml. of distilled water and a drop of methyl red, and repeat the precipitation with sodium carbonate as before. Centrifuge and add the supernatant liquid to the second Pyrex centrifuge tube. To this solution add, drop by drop, a measured quantity of 5 N  $\text{CH}_3\text{COOH}$  until the liquid becomes pink. Plunge the tube in a boiling water bath to remove all  $\text{CO}_2$  formed in the process. Add more dilute acetic acid, about 1 N, to keep the liquid slightly acid, if necessary. Evaporate the solution thus obtained to about 1 ml., cool, add 1 ml. of Reagent A, and proceed as directed under "Procedure." The standard should be treated in the same manner as the

TABLE IV

*Determination of Potassium on Sample of Serum from Callinectes danae Smith*

Serum corresponding to aliquot taken from solution of ash	Final volume	Cell length	D	E	k	K
ml.	ml.	cm.				mg. per 100 ml.
0.04	7.5	1	49		0.310	39.43
0.04	10	1	58		0.237	39.93
0.08	7.5	1	24		0.620	39.18
0.08	10	1	34.6		0.461	38.84
0.08	10	1	34		0.469	39.51
0.12	7.5	0.5	36.6	0.474	0.948	39.93
0.12	10	0.5	44	0.357	0.714	40.10
0.16	10	0.5	33	0.480	0.960	40.44
0.16	10	0.5	33.6	0.474	0.948	39.83
Mean						39.69
" error of each determination						0.5785
" " " mean						0.1929
Coefficient of variation						1.46

TABLE V

*Determination of Potassium on Sample of Serum from Bufo marinus L.*

Serum corresponding to aliquot taken from solution of ash	Final volume	Cell length	D	E	k	K
ml.	ml.	cm.				mg. per 100 ml.
0.04	7.5	2	57	0.244	0.122	15.42
0.04	10	3	53.5	0.272	0.091	15.33
0.04	10	3	54.5	0.264	0.088	14.83
0.08	7.5	2	34.4	0.463	0.232	14.66
0.08	10	3	30	0.523	0.174	14.66
0.08	10	3	28	0.552	0.184	15.50
0.16	7.5	1	34		0.469	14.82
0.16	10	1	43		0.367	15.46
0.16	10	1	44.5		0.352	14.83
Mean						15.06
" error of each determination						0.3605
" " " mean						0.1202
Coefficient of variation						2.39

sample solution. The color readings must be made within 10 to 30 minutes after the addition of the reagents.

TABLE VI  
Determination of Potassium on Sample of Human Serum

Serum corresponding to aliquot taken from solution of ash	Final volume	Cell length	D	E	k	K
ml.	ml.	cm.				mg. per 100 ml.
0.04	7.5	2	47.5	0.322	0.161	20.35
0.04	10	3	45	0.347	0.116	19.55
0.04	10	3	43.5	0.362	0.121	20.39
0.08	7.5	2	23	0.638	0.319	20.17
0.08	10	2	32	0.495	0.248	20.89
0.08	10	2	33	0.482	0.241	20.30
0.12	7.5	1	34		0.469	19.76
0.12	10	1	45		0.347	19.49
0.12	10	1	43		0.367	20.61
0.16	7.5	1	23		0.638	20.16
0.16	10	1	34.6		0.461	19.42
0.16	10	1	35		0.456	19.21
Mean						20.03
" error of each determination						0.5272
" " " mean						0.1522
Coefficient of variation						2.63

TABLE VII  
Recovery of Potassium Added to Serum

Serum	K corresponding to quantity of serum used in analysis	K added	K found	K recovered
	γ	γ	γ	γ
<i>Callinectes danae</i> Smith	31.75	12	42.45	10.70
	31.75	16	47.59	15.84
	31.75	20	53.26	21.51
	63.50	12	74.41	10.91
	63.50	16	81.07	17.57
	63.50	20	83.58	20.08
Human	16.02	12	27.57	11.55
	16.02	20	34.41	18.39
	32.04	12	42.26	10.22
	32.04	16	49.27	17.23
	32.04	20	51.22	19.18

### Results

According to the results shown in Tables IV, V, and VI it is easy, by exercising care, to obtain a fair degree of accuracy with this method.



The recovery of potassium added to the various sera investigated was remarkably good, considering the small quantity of potassium involved, as is shown in Table VII. To the sample a measured quantity of a standard solution of potassium was added, the whole was ashed, and the entire procedure carried out as described.

#### SUMMARY

A further modification of the silver cobaltinitrite method of Breh and Gaebler for use with 0.05 to 0.20 ml. of blood serum is presented.

Potassium is isolated as the potassium silver cobaltinitrite and cobalt is determined photometrically by employing the Chiarottino color test. The applicability of this reaction was studied for quantitative estimation of cobalt in the potassium silver cobaltinitrite complex.

The color system follows the Lambert-Beer law; therefore the concentration of an unknown is readily calculated.

The potassium content of 0.05 to 0.2 ml. of solutions containing 15 to 40 mg. per 100 ml. was estimated by the photometric determination of cobalt in the potassium silver cobaltinitrite precipitate with an error inferior to 3 per cent.

A simple, yet reliable, method of ashing the sample is described, and analyses for blood serum from different animals are given.

Good recovery of potassium added to the sera used was obtained.

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## LETTERS TO THE EDITORS

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### AEROBIC PHOSPHORYLATIONS IN TISSUE SLICES\*

*Sirs:*

In the course of studies on phosphorylative mechanisms in surviving rat kidney and liver slices, it has been found that organophosphates do not accumulate in the slices. In fact, there is breakdown of organophosphates initially present. This occurs even though the slices show considerable metabolic activity, as indicated by oxygen uptake.

When NaF is added to the medium, there is an increase in the amount of organic phosphates in the slices. It is difficult to tell whether the increase is due to an inhibition of the decomposition of the organophosphates or whether the increase represents newly synthesized esters. However, it has been possible to solve this problem through the use of radioactive phosphorus.

In the table are given the results of a typical experiment with kidney slices. In the absence of NaF, there is much less radioactive phosphorus fixed in the organic form than in the experiments in which NaF is present. This takes place despite the fact that NaF causes a depression of the oxygen uptake. This is understandable, since fluoride probably inhibits the phosphatase which normally causes the breakdown of the newly synthesized compound.

It is of importance to note that there are negligible amounts of adenosine pyrophosphate in the slices even when fluoride is present. Only from 10 to 20 per cent of the total organic phosphate contains radioactive phosphorus. The radioactivity is present largely in a compound which appears to be phosphoglyceric acid.

Glucose and lactate are not effective in increasing the formation of the intermediate. Malonate partially inhibits the fixation of the radioactive phosphorus. In the absence of oxygen, there is no accumulation of the radioactive compound (see the table). Because there is no synthesis anaerobically, it is likely that the radioactive compound is formed through aerobic phosphorylation reactions similar to those described by Colowick

\* This study was aided by a grant from the John and Mary R. Markle Foundation.

*et al.*<sup>1</sup> and Ochoa.<sup>2</sup> These experiments can be considered as evidence that aerobic phosphorylation processes occur in excised tissues.

	Per gm. tissue		Radioactivity per gm. tissue*		
	Inorganic P <sup>31</sup> mg.	Organic P <sup>31</sup> mg.	Inorganic P <sup>32</sup>	Organic P <sup>32</sup>	QO <sub>2</sub>
Without F	0.510	0.240	24.36	1.84	116
	0.622	0.358	25.18	2.32	115
	0.422	0.262	18.08	1.72	121
With F	0.450	0.650	29.45	8.55	77
	0.529	0.620	29.05	6.95	71
	0.550	0.725	31.50	7.90	80
“ “ in absence of O <sub>2</sub> (in helium)	0.800	0.400	37.15	0.650	
	0.690	0.475	36.90	0.745	
	0.735	0.384	37.41	0.800	

\* Expressed as per cent of the radioactivity originally present in the medium.

We are indebted to Professor Harold E. Himwich for his encouragement and advice in this work.

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<sup>1</sup> Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.*, **133**, 359 (1940).  
Colowick, S. P., Kalekar, H. M., and Cori, C. F., *J. Biol. Chem.*, **137**, 343 (1941).

<sup>2</sup> Ochoa, S., *J. Biol. Chem.*, **151**, 493 (1943).

## THE EFFECT OF ANTERIOR PITUITARY EXTRACT AND OF INSULIN ON THE HEXOKINASE REACTION\*

*Sirs:*

The first step in the utilization of glucose by animal tissues, a step common to its transformation to glycogen and its oxidation, is catalyzed by hexokinase: glucose + adenosine triphosphate  $\rightarrow$  glucose-6-phosphate + adenosine diphosphate. This reaction can be inhibited by anterior pituitary extract (APE),<sup>1</sup> either by injecting rats with APE prior to the preparation of tissue extracts or by adding APE to the enzyme preparation *in vitro*, and the inhibition can be counteracted by insulin<sup>2</sup> either *in vivo* or *in vitro*.

When phosphate-saline extracts of various rat tissues (muscle, liver, kidney, heart, brain) are used and APE is added, the hexokinase activity curves show a lag period, the duration of which depends on the amount of APE added. The resumption of hexokinase activity after 15 minutes of incubation, which is shown in Fig. 1, is due to an enzymatic inactivation of APE in crude tissue extracts. When purified preparations of muscle hexokinase are used, the inhibition of hexokinase by APE is not released with time (Fig. 2). In contrast to animal hexokinase, yeast hexokinase is not inhibited by APE.

Rats made diabetic by the injection of alloxan yield tissue extracts which show the same enzyme activity curves as those obtained from rats previously injected with APE. The brain is an apparent exception, since brain extracts prepared from rats injected with alloxan or with APE do not show an inhibition of hexokinase activity. Brain extracts can, however, be inactivated by addition of APE *in vitro*.

The conversion of glycogen to lactic acid in muscle extract is not inhibited by APE. When oxygen consumption is measured in a dialyzed liver dispersion, oxidation of glucose is inhibited by APE, while that of fructose-6-phosphate and of pyruvate is not.

Insulin releases hexokinase from APE inhibition in all cases mentioned, but does not by itself enhance hexokinase activity under these experimental conditions. Within a certain range the release of inhibition is proportional to the amount of insulin added *in vitro*. That the *in vivo* action of insulin is of a similar nature is indicated by the fact that muscle extracts prepared

\* Supported by a research grant from the Nutrition Foundation, Inc.

<sup>1</sup> The K fraction described by Greaves, Freiberg, and Johns (*J. Biol. Chem.*, **133**, 243 (1940)) was used in all cases.

<sup>2</sup> An amorphous insulin preparation (20 units per mg.) was supplied by Eli Lilly and Company.

from diabetic rats after the injection of insulin show normal hexokinase activity. When insulin is reduced by cysteine, it no longer exerts its antagonistic effect against APE inhibition of hexokinase activity.

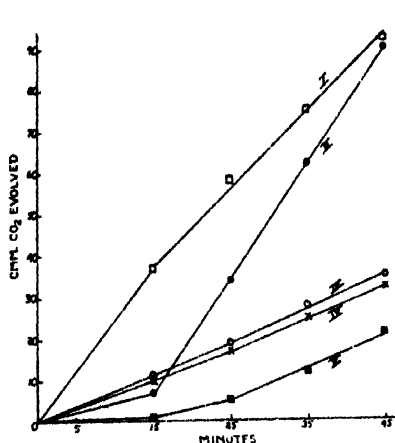


Fig. 1

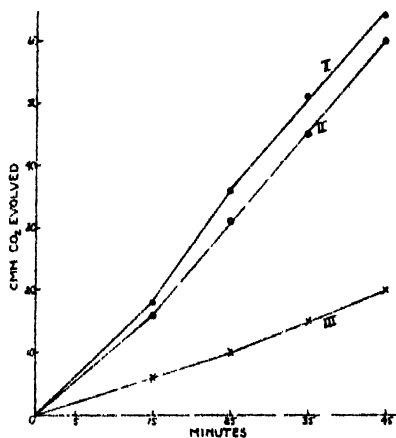


Fig. 2

FIG. 1. The effect of APE (K fraction) on the hexokinase activity of phosphate-saline extracts of liver and of APE and insulin on phosphate-saline extracts of muscle. The hexokinase activity is measured manometrically as described previously (Colowick, S. P., and Price, W. H., *J. Biol. Chem.*, **157**, 415 (1945)). APE and insulin are added from the side arm 5 minutes before tipping in the substrates. Curve I, liver hexokinase; Curve II, liver hexokinase + 200  $\gamma$  of APE; Curve III, muscle hexokinase; Curve IV, muscle hexokinase + 500  $\gamma$  of APE + 100  $\gamma$  of insulin; Curve V, muscle hexokinase + 500  $\gamma$  of APE.

FIG. 2. The effect of APE (K fraction) and insulin on purified muscle hexokinase. A fraction of fresh phosphate-saline extract precipitated by 25 per cent acetone at pH 5.9 in the cold was used. The experimental conditions were the same as in Fig. 1. The disappearance of adenosine triphosphate was determined chemically at the end of the experiment and was found to be in agreement with the results obtained by the manometric procedure. Curve I, hexokinase; Curve II, hexokinase + 400  $\gamma$  of APE + 75  $\gamma$  of insulin; Curve III, hexokinase + 400  $\gamma$  of APE.

The numerous implications of these findings will be discussed in later publications.

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## METHYLATION OF GUANIDOACETIC ACID BY HOMOCYSTINE PLUS CHOLINE WITH RAT LIVER SLICES

Sirs:

The methylation of guanidoacetic acid by liver slices is accelerated by methionine; choline, under these conditions, exerts no significant accelerating effect.<sup>1</sup> In view of the fact that homocystine plus choline can replace methionine for growth,<sup>2</sup> and of the isotope experiments which proved the transfer *in vivo* of the methyl groups of choline to creatine,<sup>3</sup> it has been suggested, from indirect evidence, that the pathway of the methyl group to creatine is more direct from methionine than from choline.<sup>4</sup> More specific evidence is desirable, especially as neither homocystine nor homocysteine has been identified in animal tissues.

In experiments with rat liver slices designed to obtain such evidence it was found that *dl*-homocystine plus choline accelerates the methylation of guanidoacetic acid as effectively as does *dl*-methionine. Homocystine is ineffective without choline, as is choline without homocystine.

*dl*-Homocystine and choline are more effective than *dl*-homocysteine and choline.<sup>5</sup>

These observations are the first, as far as we are aware, on the utilization, outside the whole animal, of the methyl groups of choline in the formation of creatine.

They furnish direct evidence that homocystine can function as a carrier of the methyl groups of choline in the methylation of guanidoacetic acid; and as it is more effective in this respect than homocysteine, the actual carrier is probably closer to homocystine than to homocysteine. The occurrence of homocystine or homocysteine *in vivo* remains to be demonstrated.

The above observations do not answer the question whether the formation of methionine is an obligatory antecedent to the methylation of guanidoacetic acid by homocystine plus choline. The immediate methyl donor to guanidoacetic acid may be methionine or a derivative of it or of methylated homocystine. We hold this question open because of observations (unpublished) on the inhibition of the methylation of guanidoacetic acid by oxidation inhibitors, *e.g.* KCN, As<sub>2</sub>O<sub>3</sub>, and As<sub>2</sub>O<sub>5</sub>.

<sup>1</sup> Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **132**, 559 (1940).

<sup>2</sup> du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).

<sup>3</sup> Simmonds, S., Cohn, M., Chandler, J. P., and du Vigneaud, V., *J. Biol. Chem.*, **149**, 519 (1943).

<sup>4</sup> du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942-43).

<sup>5</sup> We wish to thank Professor Vincent du Vigneaud for his generous gift of the *dl*-homocystine and *dl*-homocysteine used in these experiments.



*Methylation of Guanidoacetic Acid by Rat Liver Slices*

The results are expressed as  $Q_{\text{creatine}} \times 100$ . All solutions contained 1.5 mg. per cent of guanidoacetic acid.

Experiment No.	Guanidoacetic acid alone	Guanidoacetic acid plus					
		<i>dl</i> -Methionine (6.25 mg. per cent)	<i>dl</i> -Homocystine (6.25 mg. per cent)	<i>dl</i> -Homocysteine (6.25 mg. per cent)	Choline chloride (39 mg. per cent)	<i>dl</i> -Homocystine (6.25 mg. per cent) + choline chloride (39 mg. per cent)	<i>dl</i> -Homocysteine (6.25 mg. per cent) + choline chloride (39 mg. per cent)
1	1.45	5.28	1.85	2.04	2.04	5.77	2.68
2	1.72	3.19	1.61		1.87	3.46	1.50
3	1.06	3.00	0.93	0.90	1.23	2.66	1.96
4	0.39	2.01	0.40		0.32	2.46	0.80

The table summarizes some typical results. The values in the appropriate controls are omitted.

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# THE ADSORPTION OF PHOSPHATES BY ENAMEL, DENTIN, AND BONE

## II. THE ADSORPTION RATES FROM CONCENTRATED SOLUTION

*Sirs:*

The demonstrations of apparent plateaus in the adsorption of a number of ions on powdered calcified tissues after exposures of 30 to 90 minutes to the various aqueous solutions led Johansson *et al.*<sup>1</sup> recently to study

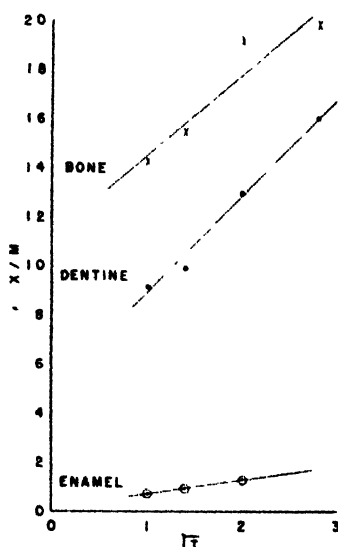


FIG. 1. Adsorption on hard tissues. Amounts of phosphates adsorbed (mg. of P per gm.) from 0.2 M aqueous solutions of disodium acid phosphate are plotted as ordinates against the square root of the exposure time in hours. Bone adsorbs more than dentin which adsorbs more than enamel. The regression lines shown have the following constants for the equation  $X/M = a + b\sqrt{t}$ : bone,  $a = 11.2$ ,  $b = 3.3$ ; dentin,  $a = 4.9$ ,  $b = 4.0$ ; enamel,  $a = 0.2$ ,  $b = 0.5$ . The lines for bone and dentin, if extrapolated, would intersect at 81 hours.

adsorption characteristics during much longer periods. Johansson *et al.* demonstrated (a) that the calcified tissues continued to adsorb ions for periods up to 64 hours with no indication of decreasing rates; (b) that diffusion was probably one of the controlling factors in the adsorption rates (shown by a linear relationship between the amount adsorbed ( $X/M$ ))

<sup>1</sup> Johansson, E. G., Falkenheim, M., and Hodge, H. C., *J. Biol. Chem.*, **159**, 129 (1945).

and the square root of the time of exposure); and (c) that although the amounts of ions adsorbed in the initial exposures were in the order bone > dentin > enamel, the slopes of the adsorption curves differed in such a way that after 64 hours exposure enamel > dentin > bone in adsorbing power.

The experiments have been repeated (Fig. 1) for periods up to 8 hours and the first two observations confirmed, although the absolute value of  $X/M$  was considerably less than that reported by Johansson *et al.* We have been unable to confirm the third observation that enamel adsorbed more than dentin or bone after prolonged exposures.

We wish to thank Miss Elizabeth Street for the calculations of the regression lines.

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